THE CLINICAL AND IMMUNOLOGICAL SIGNIFICANCE OF ECTOPIC LYMPHONEOGENESIS IN THE RHEUMATOID SYNOVIAL MEMBRANE

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Abstract

Despite the development of new biomarkers predicting prognosis in rheumatoid arthritis (RA) remains challenging and targeting of powerful biologics difficult. The presence of ectopic germinal centres (GC) within synovium has long been recognised (ectopic lymphoneogenesis [ELN]) and data have suggested that they manufacture antibody (Ab). High affinity class switched Ab production occurs through class switch recombination (CSR) and somatic hypermutation (SHM) both critically dependent on activation induced cytidine deaminase (AID). However, whether ectopic GCs express AID has not been known. Nonetheless data associating ELN with disease severity suggest a role for ELN in RA pathogenesis and as a potential biomarker. A classification system for RA synovium, based on the concept of ELN has been proposed as: (i) aggregate, (ii) aggregate GC+ and, (iii) an unorganised infiltrate. However whether these distinct pathotypes and/or degree of aggregation equate to disease severity is unclear. Thus my ***first aim*** was to develop and validate a pathological scoring system for rheumatoid synovium capable of quantifying the degree of ELN. My ***second aim*** was to investigate whether the presence and/or degree of ELN within the synovial membrane correlated with both clinical phenotype and predicted erosive damage. I demonstrate that the aggregational score developed is highly reliable and that ELN within synovial tissue associates with a higher level of synovial inflammation but is not predictive of damage.

My ***third aim*** was to investigate whether GCs within RA synovium were functional. I provide evidence of functionality by demonstrating that ectopic GCs

invariably express AID, are surrounded by anti-CCP+ plasma cells, support ongoing CSR and the manufacture of anti-CCP Abs.

My ***final aim*** was to characterise a cohort of synovial B cells consistently found surrounding ectopic GCs. I identify a novel B cell subset within RA synovium, interfollicular large B cells,(5)(5)(5) and demonstrate that interfollicular large B cells in lymph node express a somatically mutated IgH.

Declaration

I declare that the following thesis has been composed by myself, that it embodies the results of my own special work, and that it does not include work forming part of a thesis presented successfully for a degree at this or any other university.

Frances Claire Humby

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Abbreviations

A - total area of section

ACPA - anti-citrullinated peptide/protein antibodies

ACR - American college of rheumatology

AG - aggregate area within section

AIA -adjuvant induced arthritis

AID - activation induced cytidine deaminase

Anti-CCP - anti-cyclic citrullinated peptide

Anti-G6PI -anti-glucose 6 phosphate isomerase

APRIL - a proliferation inducing ligand

AP - alkaline phosphatase

AS - aggregate score

BAFF - B cell activating factor

BCMA -B cell maturation antigen

BCR - B cell receptor

BER -base excision repair

CAIA - collagen antibody induced arthritis

CII -Collagen type II

CDRs - complementary determining regions

CIA - collagen induced arthritis

CK(R) - chemokine receptor

CPJ - cartilage pannus junction

CRP - C reactive protein

CSR - class switch recombination

CTLA 4-Ig - cytotoxic T lymphocyte associated antigen

DAB - diaminobenzidine

DAS - disease activity score

DIA - digital image analysis

D -diversity

D -day

DC - dendritic cell

DS - diffuse score

EBV - Epstein Barr Virus

ELISA - enzyme linked immunosorbent assay

ELN - ectopic lymphoneogenesis

ESR - erythrocyte sedimentation rate

FACS - fluorescent activated cell sorter

FIA - Freund's incomplete adjuvant

FDC - follicular dendritic cell

G 1,2,3 -Grade 1,2,3

GC - germinal centre

GL - germline transcripts

H -hour

H&E - Haematoxylin and Eosin

HEV - high endothelial venule

Hpfs -high power fields

HLA -human leukocyte associated antigen

HRP -horse radish peroxidase

HSP -heat shock proteins

IC -immune complexes

ICC- intra class correlation coefficient

ICC_{RM} - ICC repeated measurement

ICC_{WS} -ICC within site

ICC_{BS} -ICC between site

IgH -immunoglobulin heavy chain

IHC- immunohistochemistry

IF - interfollicular

IL (R) - interleukin (receptor)

J -joining

QAS- quantitative aggregational score

LCM -laser capture micro dissection

LIGHT- herpes virus entry mediator

LPS -lipopolysaccharides

LTβR-Ig -Lymphotoxin β-Receptor Immunoglobulin

MALT- mucosal associated lymphoid tissue

MANOVA- multiple ANOVA

MC -manual counting

Mc- macrophage

MIF- macrophage inhibitory factor

MMPs -metalloproteinases

MMR- mismatch repair

MPP- medial perimeniscal

MRI- magnetic resonance imaging

mSG -minor Salivary gland

MZ -marginal zone

OCT -optimal cutting temperature

OPG -osteoprotegerin

PADI4- peptidyl arginine deiminase type 4

PAMPs- pathogen associated molecular patterns

PBMC -peripheral blood mononuclear cells

PDCD-1- programmed death receptor 1

PRR- pattern recognition receptors

pSS -primary Sjogren's syndrome

PTPN22 -protein tyrosine phosphatase N22

QT-PCR -TaqMan Real time PCR

RA -rheumatoid arthritis

RAG- recombination activating genes

RANK-L -Receptor activator of nuclear κβ-Ligand

RCTs -randomised control trials

RF -rheumatoid factor

ROI- region of interest

S -switch

SCID -Severe combined immunodeficiency

SCID-HuRA -SCID Human RA mouse chimera

SDF-1-stromal cell derived factor-1

SE -shared epitope

SHM -somatic hypermutation

SLE -systemic lupus erythematosus

SNP -single nucleotide polymorphism

SPP -suprapatellar pouch

sSS -secondary Sjogren's syndrome

STAT4 -signal transducer and activator of transcription 4

TACI -transmembrane activator and calcium modulator and cyclophilin ligand interactor

TCR -t cell receptor

Th -T helper cell

TNF- tumour necrosis factor

TLRs -toll like receptors

TLS -tertiary lymphoid structures

UK- United Kingdom

USA- United States of America

V -variable

VGEF -vascular growth endothelial factor

Chapter 1: Introduction

History

Rheumatoid arthritis (RA) was first described by Landre-Beauvais in 1800 as "primary aesthenic gout", a disease made distinct from traditional gouty arthritis by a preponderance in women, a chronic course, systemic symptoms and a lack of uric acid crystals within joints(7;8). The term RA was first coined by Garrod in his text published in 1859, but its classification as a disease distinct from osteoarthritis was not finalised until 1907.

There is little evidence to suggest that RA existed much earlier than 1800 in Western Europe(9). However evidence of its existence in the USA 6500 years ago has been demonstrated(10) with skeletons dating back 6500 years displaying a symmetrical erosive polyarthritis. This appearance of RA in Western Europe coinciding with the discovery of the New World in the fifteenth century suggests disease transmission and thus an environmental aetiology to disease pathogenesis(8).

Furthermore RA is the first autoimmune disease to be described, with the identification of rheumatoid factor (RF) in the serum of patients reported by Waaler in 1940(11).

Definition and Classification

RA is a chronic autoimmune inflammatory arthritis, characterised by the presence of inflamed synovium, loss of articular cartilage, associated bone erosion and in around 70% of patients the presence of autoantibodies in the serum. Arthritis is symmetrical, of a polyarticular form and deforming. Diagnosis is according to the 1987 American College of Rheumatology (ACR) revised criteria (12)(Table 1) that are able to differentiate RA from other forms of inflammatory arthritis with a sensitivity of around 90%. However they are less useful in those patients in

remission(13;14) and therefore are of less value in prevalence studies where diagnosis of inactive disease is required. As the emphasis on diagnosis has switched to the early diagnosis of disease, when many of these criteria may not be present, their usefulness in this cohort of patients is also limited, and so this has led to the development of new models for the diagnosis of early RA, using such criteria as anti-CCP positivity (15).

Epidemiology

RA is the commonest form of inflammatory arthritis affecting between 1-2% of the adult population worldwide. In the UK disease prevalence is 0.8%, a figure that has been consistently shown to be falling(16;17) predominantly within the female population. The annual UK incidence of disease is around 14 per 100 000 men and 36 per 100 000 women annually(18). It has a female to male ratio of 3:1, a ratio that falls with increasing age(18). The peak age of onset is in the fifth to sixth decades(19).

Several studies of prevalence and incidence of RA have been published in the last decades, suggesting considerable variation in disease occurrence between population groups. Such comparisons are limited somewhat by methodological differences adopted by investigators, these include diagnostic criteria, which changed in 1987 when the previously used 1958 criteria were reviewed, and so earlier studies may have included cases not corresponding to the current definition of RA. The use of the 1987 revised criteria however, has led to standardization and has revealed some characteristic trends seen in different ethnic populations that could be seen as independent from methodological issues.

Geographical variation in disease prevalence has been marked with lower incidences in parts of China (0.2-0.3) than in Western Europe and the USA(20),

and lower prevalence rates in Southern than Northern European countries(21-23). Further, prevalence rates of up to 5.3-6% in Native Americans and Alaskans have been noted, perhaps explained by HLA associations. Interestingly, although RA is virtually undetectable in parts of Nigeria(24) and rural South Africa(25), prevalence rates rise to nearly 1% in black populations in urban areas further suggesting an environmental aetiology to disease pathogenesis.

Clinical features

RA presents as the gradual onset of joint stiffness, pain and swelling over the course of weeks or months, although less often can present more dramatically over 24-48 hours, as an explosive onset. As RA is a systemic disease symptoms are not limited to joint involvement so in addition to fatigue, patients can present with a number of extra articular features (Table 2).

RA has a variable clinical course, with differences in radiographic joint damage varying some 10-12 fold 10 years after diagnosis(26), this is of particular importance as radiographic damage increasingly explains functional disability as disease progresses(27). Further, RA patients experience increased mortality predominantly explained by an excess in cardiovascular disease(28).

The chronic progressive nature of RA and associated morbidity and mortality result in a health economic burden in the UK alone of £1.2billion annually.

Table 1: 1987 revised American College of Rheumatology criteria for diagnosis of rheumatoid arthritis.

1)*Morning stiffness in and around joints lasting for at least an hour before maximal improvement

2) *soft tissue swelling (arthritis) of 3 or more joint areas observed by a physician

3)*swelling (arthritis) of the proximal interphalangeal, metacarpophalangeal, or wrist joints;

4) *symmetric swelling (arthritis)

5) rheumatoid nodules

6) the presence of rheumatoid factor;

7) Radiographic erosions and/or periarticular osteopenia in hand and/or wrist joints.

**Criteria 1 through 4 must have been present for at least 6 weeks. Rheumatoid arthritis is defined by the presence of 4 or more criteria.*

Table 2: Extra articular manifestations of rheumatoid arthritis

Organ	Manifestation of involvement
Skin	Vasculitis, nodules
Cardiac	Pericarditis, Myocardial fibrosis/granulomatous disease
Pulmonary	Interstitial pneumonitis, Rheumatoid Nodules, Serositis
Neurological	Mononeuritis multiplex, peripheral neuropathy
Haematological	Anaemia, Felty's syndrome
Ophthalmological	Scleritis, eipscleritis, keratoconjunctivitis sicca
Exocrine	Secondary Sjogren's syndrome
Other	Amyloidosis, systemic vasculitis, osteoporosis

Aetiology

Genetic Factors

The contribution of genes to the development of RA was initially proposed at the beginning of the 20th century when the disease was noted to be prevalent in particular families. This observation led to further, more detailed studies later in the century which focused on the increased risk posed if a first degree relative was affected by RA. The conclusions were that the risk of developing RA was increased between 2 and 4 times if a first degree relative was affected. However more debate has surrounded the relative contribution of genetic factors to disease susceptibility rather than disease severity *per se*(29).

The finding that concordance rates for RA are higher in monozygotic (12-15%) as opposed to dizygotic twins (3-4%) and higher in dizygotic twins than the general population provides further evidence suggesting a genetic component to disease(30;31), but also supports the role of an environmental trigger. Extension of this work has shown that as much as 60% of a population's predisposition to RA can be accounted for by genetic factors(32).

Human Leukocyte Antigen (HLA) associations

The association between HLA genes and RA have been known for 30 years(33;34) and around 30% of disease susceptibility can be accounted for by genes within this region(35), particularly the class II gene HLA-DRB1 located on the short arm of chromosome 6(6p21.3)(32;36). HLA-DRB1 alleles associated with disease susceptibility across different ethnic groups encode a conserved sequence of amino acids in the third hypervariable region of the β chain of the HLA-DR molecule and are collectively referred to as the shared epitope (SE) (35), and the

association commonly defined as the SE hypothesis(35). These residues are important as they occur within the antigen binding site of the MHC molecule and so can influence efficiency of antigen presentation to T cells.

HLA DR4 subtypes, such as Dw10 and Dw13(37), have also been negatively associated with the development of RA, an association thought to be a result of either altered antigen binding site efficiency or by the induction of regulatory T cell pathways independent of peptide.

In addition to disease susceptibility the SE also appears to influence disease severity with specific sequences associated with a poorer prognosis(38) and heterozygosity or homozygosity for certain alleles, for example DRB1 *0401/0404, carrying a risk for more severe disease(39).

The SE hypothesis does not appear to be an essential aetiological factor however as it does not apply to all ethnic groups, with greater than 90% of Caucasian RA patients carrying HLA-DRB1 alleles and less than 50% of black Americans (40).

Recent data have demonstrated a gene-environment interaction with RA patients who both smoke and express the SE at much higher risk of sero positivity for RF and to a larger extent anti-CCP antibodies(41).

Non HLA genetic associations

Protein tyrosine phosphatase N22 (PTPN22)

Of the non HLA genetic associations a polymorphism within the gene PTPN22 has been most reproducibly demonstrated over a number of studies for both RA and a range of other autoimmune conditions (42-46). PTPN22 encodes an intracellular protein lymphoid tyrosine phosphatase a negative regulator of T cell activation, the risk allele therefore permitting MHC II dependent T cell activation. The risk (T) allele has been associated in past studies with an odds ratio for RA of

approximately 1.8 (44), and the risk appears to be amplified in autoantibody-positive RA(47-50) and in patients homozygous for the variant T allele(48). In addition, a more recent report documents a multiplicative effect with smoking(51).

Peptidyl deiminase type 4 (PADI4)

The peptidyl deiminase type 4 gene encodes an enzyme involved in the post translational modification of proteins converting arginine to citrulline. An association with disease susceptibility was suggested by a study of Japanese RA patients(52). However, subsequent reports in Caucasian patients did not replicate the findings(53-55) but a more recent meta-analysis has suggested that this may reflect an association limited to patients of Asian, rather than European origin(56). The susceptibility haplotype is thought to stabilise PADI4 mRNA and therefore increase the cellular levels of PADI4 protein and citrullinated peptides. Despite this, no consistent association between PADI4 haplotypes and circulating anti-CCP antibodies has so far been demonstrated(52;54;57).

Cytotoxic T lymphocyte associated antigen 4 (CTLA4)

An association with RA and polymorphisms within the gene CTLA4 have been reported(49). In addition CTLA4 has been shown to be associated with a number of other autoimmune diseases such as type 1 diabetes, multiple sclerosis and autoimmune thyroid disease(58;59). CTLA4 is a negative regulator of T cell activation that is rapidly up regulated following T cell activation. CTLA4 binds the molecules CD80 and CD86, both of which are expressed by antigen presenting cells and in the absence of CTLA4 bind to CD28 which functions to provide co-stimulatory signals to T cells and thus T cell activation (60). Polymorphisms for CTLA4 have been associated with an increased risk of RA in China and North America (49;61) although not in other populations (62-64). This discrepancy may

be explained somewhat by different polymorphic variants associating with Asian(65) and North European populations(49). Interestingly, further stratification of patients has also revealed an increased risk for RA in certain populations only in association with anti-CCP(49) antibodies and specific HLA subgroups(66;67).

Macrophage inhibitory factor (MIF)

MIF is a pro inflammatory cytokine expressed in a wide variety of cells including T and B cells and macrophages. Raised levels of MIF have been detected within both the serum and synovial fluid of RA patients and anti-MIF animals have demonstrated efficacy in delaying the onset of arthritis in animal models of disease(68). The first association of genetic polymorphisms within this gene with inflammatory disease was with juvenile inflammatory arthritis(69) and identical polymorphisms as well as additional variants have now been correlated with RA(70). However further studies have produced conflicting results with a large cohort study from Sweden and the USA which showed no association with RA (49), and differing reports of associations of specific polymorphisms of MIF with disease severity(70-72).

Signal transducer and activator of transcription 4 (STAT4)

A SNP haplotype in STAT4 has recently been associated with RA in a North American population(73;74) with an odds ratio of RA associated with the risk allele of 1.32. This association has been further replicated in Swedish and Korean cohorts(74;75). STAT4 encodes a protein expressed primarily by lymphoid and myeloid tissues(76). Although how the RA associated STAT4 haplotype affects STAT4 production is currently unknown, it is conceivable that as STAT4 plays a central role in the cellular responses to a number of cytokines including IL-12 and

IL-23. This may result in a skewed Th1/Th2 response via IL-12 or influence via Il-23 a pro inflammatory Th17 response(76).

6q23

The 6q23 locus has been identified in a number of genome wide studies from different populations as a risk factor for RA(77-79). The single nucleotide polymorphism (SNP) maps close to the TNF-α-induced protein 3 (*TNFAIP3*) (78;79) and as its product is capable of down regulating inflammation mediated by TNF-α and by Toll-like receptors(80;81) may well play a role in RA pathogenesis.

TRAF5-C1

This has also been identified in a genome wide study(82) and is a plausible candidate for RA pathogenesis as its product acts to negatively regulate T cell receptor and TNF receptor signalling(82). Further C5 as a critical component of the complement pathway is thought to play a role in mediating articular damage in RA.

Programmed death receptor 1 (PDCD-1)

PDCD-1 was first isolated in 1992; it is a molecule whose expression is enhanced by apoptotic stimuli. PDCD-1 is thought to prevent autoimmune diseases, including RA, by inhibiting activation of self reactive lymphocytes. Polymorphisms of the PDCD-1 gene have now been linked to a number of immune mediated diseases including RA(83-85), although the association does not appear to persist in all ethnic groups(86).

Environmental factors

Infectious agents

A search for an infectious trigger for RA has been ongoing for many years and a number of candidates have been proposed including mycoplasma (87-90), mycobacterium tuberculosis(91), Human retrovirus 5(92), alpha virus(93), Epstein-Barr virus (EBV) (94;95), cytomegalovirus, rubella virus (96)and parvovirus B19(97;98).

Parvovirus B19 has attracted much attention(99) not least as clinical infection is often accompanied by an arthritis that is initially clinically indistinguishable from RA, with a symmetrical inflammatory arthritis, sero positivity for RF and bony erosions. In addition, the detection of parvovirus B19 DNA in RA synovial tissue and fluid as well as data demonstrating an ability of B19 positive sera to transform normal synoviocytes into an invasive phenotype(100) have further supported its case as a putative trigger. More recent studies have not supported this role, however, with a prospective study of acute parvovirus B19 arthritis patients showing no development of chronic arthritis(101) and further data demonstrating detection of its DNA in the synovial tissue of healthy individuals(102).

EBV has also been implicated in RA pathogenesis with the detection of high titre antibodies in the sera of RA patients(103). Interestingly the EBV glycoprotein gp110 shares amino acid homology with the protein encoded by the RA susceptibility associated HLA-DRB*0401 allele. This allele is thought to confer low T lymphocyte responses to gp110 and thus allow abnormally high EBV replication in specific RA patients(104). EBV DNA has further been detected in significantly higher amounts in the blood, synovial fluid and saliva of RA patients

than in healthy controls and patients with other inflammatory arthritidies and EBV encoded RNA detected in B lymphocytes from synovial tissue (reviewed in(95)). The remaining organisms have all been proposed as putative pathogens in RA but links are controversial. Indeed at present there appears to be no consistent evidence to suggest that a single infectious agent is responsible for RA pathogenesis, although many avenues of research are investigating such a possibility.

Lifestyle factors

Smoking

Smoking has for some time been known to be the major epidemiological risk factor for the development of RA(105-107) and some evidence supports an association with disease severity(108;109). The increased risk is seen particularly in RF positive and anti-CCP positive patients. Importantly in anti-CCP positive patients the combination of the presence of the SE and smoking increases the relative risk of developing the disease by 21 fold, if 2 copies of the gene are inherited, compared with non smokers without the risk gene (110). The explanation for the link between smoking and the presence of anti-CCP antibodies was proposed when an increased presence of citrulline modified proteins were noted in the lungs of smokers. This was particularly interesting as it was known that the citrullination of proteins appeared to confer affinity for binding of their peptides to the SE of the HLA-DR molecules(111). Thus it was proposed that long term smokers increased the conversion of arginine to citrulline in the lung by upregulation of enzymes such as peptidyl-arginine deiminase (PADI) in macrophages(112). In those individuals carrying the SE an immune response would be preferentially be established, considering the increased affinity of

binding of citrullinated peptides for the SE, and the adaptive immune system activated some years before the development of the disease.

Obesity and diet

Obesity has been found to be a risk factor for RA in female, but not male patients(113;114). Diet has also been demonstrated to influence symptomatology where diets rich in omega- 3 fatty acids found in oily fish were associated with improved pain scores (115), decreased use of NSAIDs(116) and modest clinical improvements(117). These effects are thought to be mediated by direct competition of fatty acids with the pro inflammatory arachidonic acids. Conversely a diet rich in red meat has been linked to an increased risk of RA(118). Finally, an increased intake of vitamin D has been associated with a reduced risk of developing RA(119).

Hormonal and reproductive factors

The higher incidence of RA in women, particularly prior to the menopause, suggests a role for hormonal factors in disease pathogenesis, which is further supported by the observed improvement in RA concurrent with pregnancy. The exact mechanism for each of these effects is not clear. A widely held view however that male RA patients have lower levels of testosterone was not supported by a study showing that levels of androgen-anabolic hormones, such as testosterone, are not significantly altered in RA patients compared to controls(120). Data have also suggested a particular clinical phenotype for each sex, with male patients being associated with a more erosive accelerated disease course in one study(121), however this observation was not been supported by a later report (122). Exposure to exogenous hormones in the form of the oral contraceptive pill does appear to have a protective effect, an observation reported

by a number of groups(123;124). However hormone replacement therapy does not appear to influence disease(125). The molecular mechanisms governing these effects are still to be elucidated.

The beneficial effect of pregnancy on RA has been suggested to be a result of a number of factors, including HLA class II disparity between mother and foetus(126) and quantitative and qualitative changes in regulatory T cells during pregnancy and post partum (127). Pregnancy *per se* also appears to increase the risk of developing RA, an effect particularly noticeable during the immediate post partum period(128;129) particularly in breast feeding mothers, thought to be due to a result of prolactin(130).

Weather conditions

Although long regarded as an old wives tale there has been some recent data to support an influence of prevailing atmospheric conditions on symptomatology, with 2 groups reporting an association of joint pain with cold weather(131;132).

Current prognostic markers in rheumatoid arthritis

The overwhelming balance of expert opinion now favours the use of aggressive treatment in early RA(133;134)in an attempt to induce remission before erosive damage becomes irreversible, as it is this progression of erosive damage that has been reported to explain the loss of function seen in patients over the course of the disease (135). Two recently reported studies(133;134) have achieved rates of remission of around 50% using biological agents in early RA and confirmed the concept of an available window of opportunity early on in disease to induce remission, which in the absence of a cure is the best possible outcome. RA, however, has a very variable clinical course, and not all patients have a poor outcome, scores for joint damage varying 10-12 fold after 10 years of disease(136)

and importantly in 10% of patients severe joint damage with significant associated morbidity occurs within 2 years of diagnosis(137). Treatment ideally should be tailored on an individual patient basis, maximising treatment to those most likely to have the most severe course. Giving all patients with early RA maximal treatment would not only incur high costs but the substantial risks of side effects, which include an increased risk of infection(138), would be unacceptable. Further, current ACR classification criteria have been shown only to have diagnostic sensitivity and specificity in established RA and to miss a significant proportion of patients with early RA(139). Thus there has been a need to develop reliable prognostic markers capable of identifying those patients with early arthritis who are going to progress rapidly.

A large amount of research has focused in recent years on an attempt to identify genetic prognostic markers. Among all the HLA-DRB1 alleles examined HLA-DRB1*0401 and DRB1*0404 have been consistently associated with radiographic erosions and moreover, the effect appears to be dose dependent with patients with 2 disease associated alleles having more erosions and more joint replacement surgery than those without(140;141). Further, genes within the HLA region that have been associated with erosive progression are HLA-DMA*0103 in combination with HLA-DRB1*01(142). Further protective genes have also been identified, particularly polymorphisms of the HLA-DRB1 and uteroglobin genes, associated with a lower risk of erosions(143;144). However, so far no genetic test is currently of use in clinical practice.

Disease activity at onset has been shown to be predictive of poor prognosis, in particular high levels of ESR and CRP(145;146). The health assessment

questionnaire also predicts outcome, although disability rather than erosive damage(147;148).

RF has for many years been considered as a useful prognostic factor and numerous studies have identified positivity along with high titres and IgA subclass as associated with a poorer outcome(145;149-151). Anti-CCP antibodies have now been shown to have a superior specificity and similar sensitivity to RF in the diagnosis of RA, and moreover also predictive of erosive damage in early arthritis(146;152).

Radiographic damage at baseline has been shown to be strongly predictive of further erosive damage by a number of studies(145;146;153), however its use as a prognostic tool is limited by a lack of erosions at disease onset in the majority of patients. Thus the use of both magnetic resonance imaging (MRI) and ultrasonography have been explored in an attempt to quantify erosive disease before it is identified on plain radiography. Both modalities have been shown to be sensitive to the early inflammatory and erosive changes seen in early inflammatory arthritis, but neither have been shown to be unequivocally predictive of or related to radiographic changes typical of RA, or to major clinical outcomes such as long-term functional abnormalities(reviewed in(154-156). Indeed a controlled study using MRI could only detect changes in MCP bone marrow oedema in patients with RA, in comparison to Sjogren's syndrome or systemic lupus erythematosus (SLE), and in particular erosive changes did not differ between patient groups (157).

Finally the ratio of circulating osteoprotegerin (OPG) to receptor activator of nuclear κβ- Ligand (RANK-L) has been demonstrated to inversely correlate with erosive damage in those patients with early RA(158).

A number of prognostic scores combining biological parameters such as ESR, CRP, RF, anti-CCP and HAQ scores to determine outcome have been developed. The most recent was a model proposed by Visser et al comprising 7 variables and allowing for discrimination between persistent and self limiting arthritis and erosive and non erosive arthritis(15). Unfortunately, until now this and other scores developed have not been validated on other cohorts of arthritis so do not currently form part of standard clinical practice.

Immunopathology

The core of RA pathogenesis is generally considered to be the result of a dysregulated immune system with multiple interactions between the innate and adaptive immune systems resulting in an inflammatory cascade.

Innate Immunity

The innate immune system provides a rapid first line defence mechanism against microbial pathogens. Elements of the innate immune system include anatomical barriers, secretory molecules and cellular components. Anatomical barriers include mucosal layers and epithelial cell shedding at surfaces continuously exposed to micro organisms such as is found in the skin, gut and lungs. In addition to these mechanical factors barriers also produce factors capable of inhibiting bacterial growth, such as lysozymes and phospholipases found in the salivary glands and fatty acids in sweat. Finally biological mechanisms such as "bacterial flora" exist on the skin and in the intestine that prevent the colonisation of the sites with pathogenic organisms. Cellular and secretory mechanisms of innate immunity are activated following the recognition of pathogen-associated molecular patterns (PAMPs) expressed by the majority of micro organisms, by germ line encoded pattern recognition receptors (PRR). PRPs include lectins, mannose-binding

receptors, dectin-1, DEC-205, pentraxins, NOD proteins and toll like receptors (TLRs) and are all expressed to a varying degree by cells of the innate immune system. The activation of PRR by PAMPs has a number of different outcomes dependent on the receptor and cell type that includes complement activation and opsonisation; phagocytosis and clearance of pathogens and cellular debris, antimicrobial peptide superoxide, nitric oxide, pro inflammatory cytokine and chemokine release and the upregulation of co stimulatory molecules. This production of proinflammatory cytokines and chemokines and the upregulation of co-stimulatory molecules in particular allows TLRs to provide a crucial link between the innate and adaptive immune systems. It has therefore been proposed that dysregulated TLR signalling may provide a pathway to autoimmunity(159). The role of TLRs in RA pathogenesis has thus received much interest and this concept is supported by the observation that TLR signalling appears to play an important role in animal models of arthritis such as streptococcal wall arthritis(160). A more recent and crucial advance has been the demonstration that chromatin activation of B cells via synergstic engagement of B cell receptors (BCR) and TLR9 can result in the production of autoantibodies(161). Furthermore both TLRs, in particular TLR2, TLR3 TLR4 and TR7(162-164)and receptor ligands, including heat shock proteins, hyaluronan and fibrinogen have been detected within the RA joint. Stimulation of these receptors on synovial fibroblasts has now been demonstrated to be associated with the production of a number of pro inflammatory cytokines and metalloproteinases(165) (166;167). This association provides a mechanism by which innate immunity can be actively involved in RA pathogenesis and indeed blockade of TLR signalling is being explored as a possible therapeutic option(168).

Adaptive immunity

In contrast to the non specific innate immune response adaptive immunity is highly specific. It is mediated by both cellular and humoral immunity, a result of clonally expanded T and B cells and forms the basis for immunological memory.

T cells

αβT cells can be divided into two subgroups dependent on the expression of CD4 and CD8, known respectively as T helper (Th) cells and cytotoxic T cells. $CD4^+$ T cells interact with MHC class II molecules bearing antigen expressed on antigen presenting cells such as dendritic cells or B cells. This interaction enables the Th cell to release cytokines, capable of a number of functions including providing stimulation to B and T cells to proliferate and attracting other immune cells such as macrophages. $CD8^+$ T cells in comparison act via MHC I directed antigen specific killing of target cells and via the production of cytokines, particularly IFN-γ and TNFα.

Classically naive Th cells have been thought capable of differentiating into 2 possible lineages, Th1 and Th2. Th1 cells are known to secrete predominantly IFNγ and lymphotoxin and are responsible for clearing intracellular pathogens by activating macrophages(169). Similarly, Th2 cells secrete IL-4, IL-5, IL-13, IL-25 and help in mediating immunity against extracellular pathogens with the production of IL-4 and IL-5 in particular resulting in the activation of eosinophils and mast cells(170). The cytokines of a specific lineage have also been shown to both operate a positive feedback loop promoting further proliferation of that specific subtype whilst simultaneously inhibiting proliferation of the other subsets. Thus, while Th1 cells induce pro-inflammatory responses such as delayed-type hypersensitivity, Th2 cells are capable of mediating allergic reactions.

RA was long thought of as a Th1 mediated disease until experiments demonstrating that the loss of IFNγ did not prevent the onset of autoimmunity (171;172)suggested that other pathways outside Th1 may play a role in disease pathogenesis . It is now known that a third Th cell lineage exists, that of Th17, promoted by TGFβ, IL-6 and IL23(173-175). Importantly each of these separate Th lineages has inhibitory regulatory effects on the others. Th17 cells have now shown to be a critical pathogenic component of a number of autoimmune diseases including RA, with demonstrated expression within the RA synovial membrane (176)and early studies on animal models of arthritis suggesting that IL-17 inhibition maybe a viable therapeutic option(177). A further and interesting finding has been the reciprocal relationship of Th17 cells with regulatory T cell production, mediated in part via an inhibitory effect of IL-6 (178).

B cells

After birth B cells develop in the bone marrow and as soon as they have productively rearranged their immunoglobulin genes they then migrate to the periphery to mature within the spleen into long lived mature B cells (Figure 1).

Transitional B cells

Transitional B cells are functionally immature and can be characterised by their short half lives, their propensity to dominate the early phases of B cell reconstitution and their capacity to undergo apoptosis rather than proliferation following BCR engagement(179). They can be subdivided into three divisions (Figure 1), transitional (T) 1, T2 and the more recently recognised T3 subtype (180). T1 cells are IgMhighCD23- , T2 cells are IgMhigh CD23+,and T3 cells IgMlow CD23+(180). Transitional B cells develop following migration from the bone marrow into either B1 or B2 naive B cell subsets. B2 cells comprise follicular

and marginal zone B cells and are thought to develop from T2 B cells. The origin of B1 B cells is less clear, although as they are lacking in splenectomised animals and as the spleen is the site of maturation of T2 and T3 cells, they are thought likely to develop from T2 or T3 cells(181). T3 B cells, although once thought to be part of the linear development from immature to mature B cells, are now thought to represent primarily self-reactive anergic B cells(182) and thus play a role in the development of peripheral tolerance.

Marginal Zone (MZ) B cells

MZ B cells are known to be able to function in both T dependent and T independent immune responses. They have been demonstrated to be capable of antibody production following TLR stimulation via differentiation in to short lived plasma cells, following their migration out of the marginal zone to the red pulp zone of the spleen(183) and are thus involved in the early non specific innate response.

MZ B cells are also able to deliver antigen to follicular B cells and thus stimulate a GC response. MZ B cells may also become involved in T dependent immune responses via their direct activation by T dependent antigens, and thus subsequently receive T cell help(184). Indeed the observation that MZ B cells express higher levels of MHC II molecules than follicular B cells makes them more efficient at presenting antigen to T cells(185).

The fate of T2 B cells to differentiate into either follicular or MZB cells has so far not been settled, although 3 possibilities are debated. Firstly, the MZ or GC fate may be determined by the quality of BCR-evoked signals and the subsequent expression of Notch proteins(186), alternatively MZ B cells have been suggested to have gone through a GC response, as they lack expression of AID and have

mutated Ig (187). Finally the expression of sphingospine-1 phosphate receptor 1 on the B cells may overcome the CXCL13 recruiting capacity to the GC(188).

B1 B cells

B1 B cells have been primarily identified within mice, and as of yet no population has been clearly identified in humans. In mice both B1a and B1b subsets of B1 B cells are known to seed the peritoneal and pleural cavities. B1 B cells are known to be of importance in generating a IgM response to T independent antigens and splenectomised mice lacking B1a B cells have a low level of natural antibodies and do not responds to immunization with T independent polysaccharides(181). Although B1a B cells clearly contribute to innate-like immune responses, B-1b B cells are thought capable of contributing to the adaptive immune response(189;190). It has been suggested that they may originate from a specialized B2 B cell in a T independent manner(190).

Follicular B cells

Once follicular B cells mature they attain the ability to recirculate in the spleen (or indeed in the bone marrow) and then migrate through the peripheral blood and lymph continuously to the B cell areas of lymph nodes, Peyer's patches and the spleen. It is within these B cell rich follicular niches that B cells can then present antigen to T cells and thus mediate T cell dependent immune responses to protein antigens. B cells are additionally recognised to receive activation signals via TLR activation, but unlike MZ and B1 B cells require additional signals from BCR and CD40 to mature into antibody producing cells(191). Activated follicular B cells can produce short lived plasmablasts that do not migrate and are part of the early T cell dependent immune response. This is in contrast to activated germinal centre B cells that can differentiate into plasmablasts that then migrate to the bone marrow

to become long lived plasma cells, sustained by the actions of APRIL and BAFF(192).

A second niche for follicular B cells is within perisinusoidal niches in the bone marrow. These perisinusoidal B cells have been demonstrated to be able to respond to T independent antigens, via the production of IgM, but they cannot induce the expression of AID and thus are unable to produce high affinity antibodies(193).

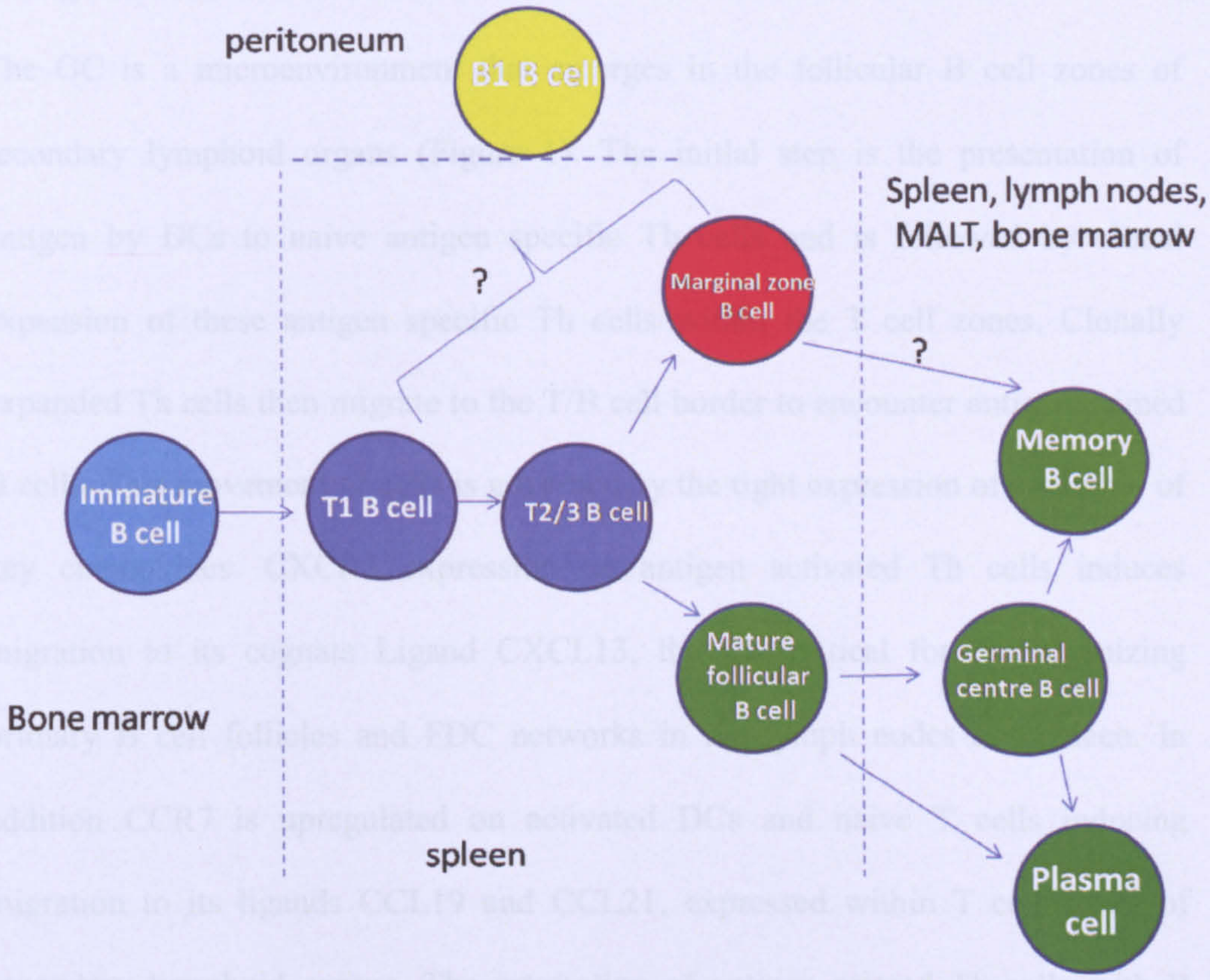


Figure 1: Stages in B cell maturation

B cell lymphopoiesis occurs exclusively in the bone marrow and immature cells transit to the spleen for final maturation. Follicular (mature), germinal centre, and plasma B cells compose the B-2 lineage. A second lineage found in the peritoneum is called B-1 (so far only identified in mice). The presence of this cell requires the spleen, which suggests possible derivation from the transitional or marginal zone B cell stage, or perhaps the splenic microenvironment is required for B-1 lineage maturation. Germinal centre formation leading to memory and plasma cells can occur in the spleen, lymph nodes, or mucosa associated lymphoid tissue (MALT). Plasma cells disperse and are found in the bone marrow, peripheral tissues, and spleen. Adapted from(194)

The germinal centre reaction

The GC is a microenvironment that emerges in the follicular B cell zones of secondary lymphoid organs (Figure 1). The initial step is the presentation of antigen by DCs to naive antigen specific Th cells and is followed by clonal expansion of these antigen specific Th cells within the T cell zones. Clonally expanded Th cells then migrate to the T/B cell border to encounter antigen primed B cells. This movement of cells is governed by the tight expression of a number of key chemokines. CXCR5 expression on antigen activated Th cells induces migration to its cognate Ligand CXCL13, the CK critical for the organizing primary B cell follicles and FDC networks in the lymph nodes and spleen. In addition CCR7 is upregulated on activated DCs and naive T cells inducing migration to its ligands CCL19 and CCL21, expressed within T cell zones of secondary lymphoid organs. The interaction of antigen primed Th cells with B cells requires antigen primed B cells to have processed and presented antigen in the form of MHC II molecules, further co-stimulatory signals are provided by CD28/CTLA4Ig and CD80/86 interactions, as well as those between inducible co-stimulator (ICOS) and B7-h on resting B cells. This T/B cell interaction is followed by the clonal expansion of antigen specific B cells, either within the T cell area and resulting in the production of short lived plasma cells producing non class switched IgM or within primary follicles within the B cell zones. At about day 7-10 following initial antigen priming the resultant secondary follicle that has developed polarizes into a dark and a light zone, containing rapidly dividing and quiescent B cells. This is the GC reaction. Rapidly expanding cells are known as centroblasts and undergo diversification of their antigen receptor via SHM within the dark zone. This process results largely in death of the resulting centrocyte

within the light zone, as antigen binding is impaired, dying cells are then cleared via tingible body macrophages. Occasionally mutations are introduced that result in increased antigen binding of the receptor for antigen. In this event the centrocyte will receive survival signals

and either remain within the GC and re enter the GC cycle, or exit as a long lived memory cell. Central to this process are FDCs, maturation of which require TNF and LTβ as evidence suggests that FDCs provide proliferative signals to centroblasts(195). Complement receptors are known to trap antigen on FDCs that are then used to test centrocyte affinity for retained antigen. The GC reaction is known to persist for approximately 21 days following initiation. The main function is to produce memory B cells, which can be divided into long lived plasma cells, that reside mainly in the bone marrow and committed memory B cells that mainly act as precursors to the recall response and able to recirculate freely to locate their specific antigen.

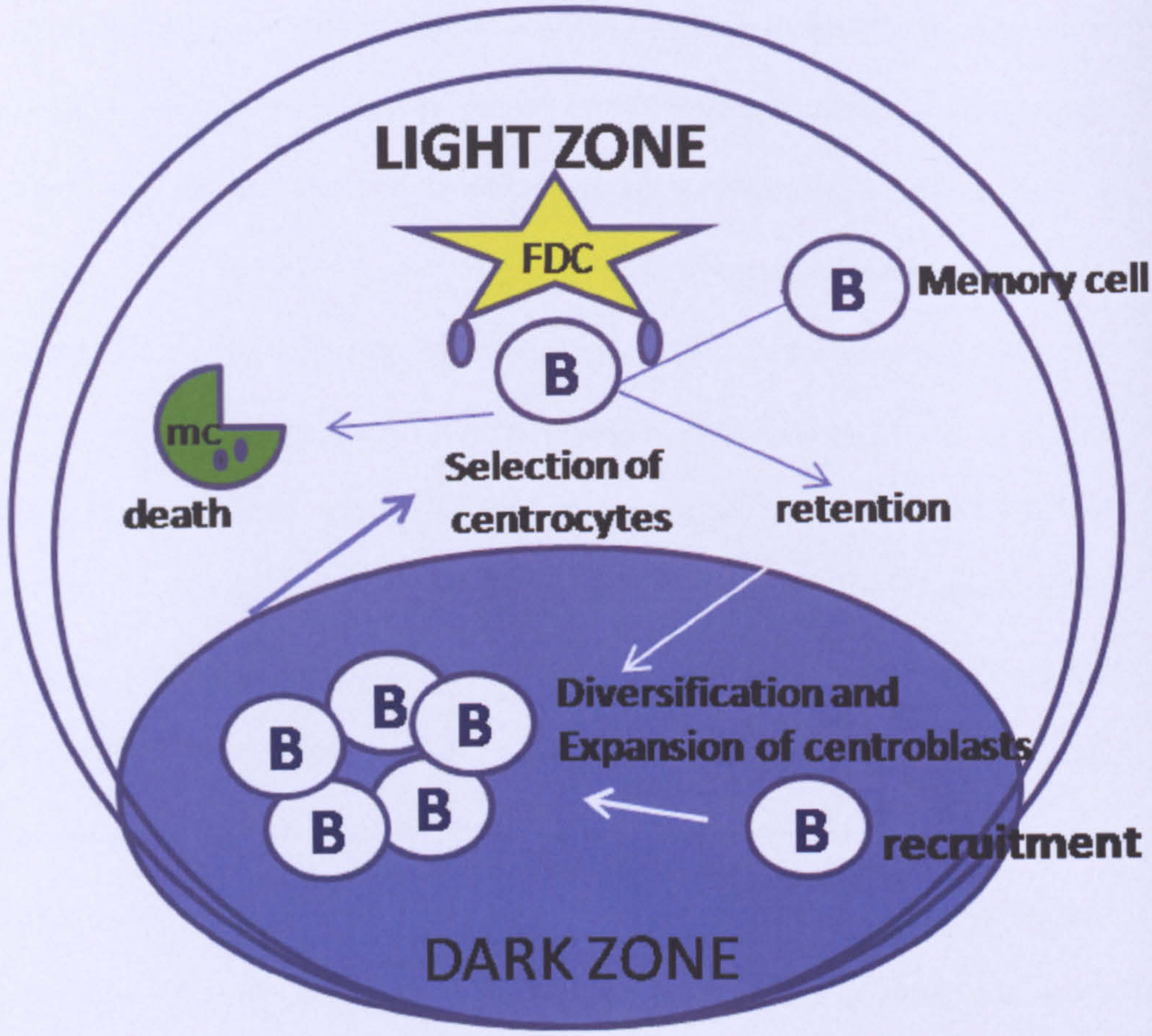
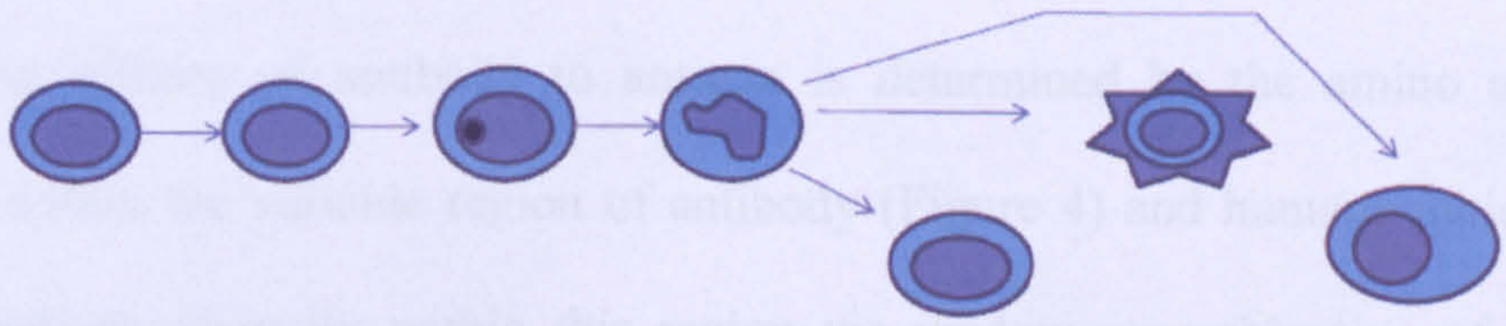

LIGHT ZONE
FDC
B
Memory cell
B
mc
death
Selection of
centrocytes
retention
B
B
B
B
B
Diversification and
Expansion of centroblasts
B
recruitment
DARK ZONE

Figure 2: The germinal centre reaction

This figure illustrates the cellular and molecular processes constituting a GC reaction. Antigen-specific B cells are recruited into the follicular regions within the B-cell zones of secondary lymphoid organs. Rapid clonal expansion results in the formation of the secondary follicle. Expanding centroblasts within the dark region are polarized from resting centrocytes within the light regions of the secondary follicle. Antigen-specific B cells diversify their BCR by somatic hypermutation and then 'test' their variant BCR for antigen held as immune complexes on follicular dendritic cells (FDCs) within the light zone. Diminished binding for antigen leads to programmed cell death and rapid clearance of apoptotic B cells locally by tingle body macrophages (mc). Improved binding to antigen results in positive selection of the variant. There are two possible outcomes. The first is to return to the dark zone and resume the cycle of expansion, diversification, and selection. The second is to exit the GC cycle and thereby enter the memory B-cell compartment.

Interfollicular (IF) large B cells

The pattern of somatic mutations within the V region genes of the IgH chain has been used to categorise B cells into either naive B cells (with unmutated V region genes) or GC/post GC B cells (with somatically mutated V region genes). A recent subpopulation of B cells, known as IF large B cells has been identified within lymph node and tonsils and found to carry somatically mutated V region genes(5). These IF large B cells have also been recognised to express AID and have thus been postulated to represent an extrafollicular GC independent pathway that could lead to the production of plasma cells(5). An alternative hypothesis, however, is that these cells represent post GC B cells, although an explanation for their continued expression of AID in this scenario is currently lacking, particularly as ongoing CSR at the interfollicular site occupied by these cells has been demonstrated(196). Indeed the molecular phenotype of these cells does not suggest that they represent errant GC B cells, as they lack expression of a number of classic GC B cell markers, such as Bcl6 and p27(5)(Figure 3). In addition, the unusual phenotype of these large IF B cells suggests that these cells may play an antigen presenting role, in that often long dendritic like processes are noted to extend some distance from the nucleus(5), although they lack the classic co-stimulatory molecules such as CD80 and CD86 associated with DCs. Thus although IF large B cells appear to represent a unique B cell population their exact origin and function is currently unknown, and whether they are expressed within ectopic lymphoneogenesis is unknown.



Cell type:	B cell precursor	Naive B cell	Centroblast	centrocyte	Memory B cell	Interfollicular large B cell	Plasma cell
Anatomical site	Bone marrow	Mantle zones	Germinal centre (dark zone)	Germinal centre (light zone)	Marginal zone	T cell rich interfollicular areas	Bone marrow lymph node medulla
morphology	Small cell	Small cell	Large round cell	Medium sized cell with irregular nucleus	Small cell	Dendritic surface nucleolus	Eccentric nucleus clockface chromatin
Molecular characteristics	CD34/TdT+	IgD/IgM+	CD10/BCL-6+		CD27+	B cell markers+ CD10/BCL6/CD27-	cIg/CD38/CD138+
Genotype	Germline Ig	Unmutated rearranged Ig	Mutated Ig with ongoing mutations		Mutated Ig		

Figure 3: Molecular characteristics of interfollicular large B cells and other B cells.

Phenotype of individual cells depicted by diagrams above table (adapted from (5))

Achieving antibody diversity

The binding affinity of antibody to antigen is determined by the amino acid sequences within the variable region of antibody (Figure 4) and humans initially achieve antibody diversity within this region via random recombination of the variable (V), diversity (D) and joining (J) region genes, a process that occurs early in B cell development and under the control of recombination activation genes (RAG) (Figure 5). It results in the production of early B cells with randomly assorted VDJ segments of genome with further variability additionally being introduced by the generation of random linking N-sequences (via the action of terminal transferase). Thus early B cells are produced with the protein product of these genes displayed on the cell surface as its cell surface antigen receptor.

When B cells subsequently come into contact with antigen two processes are initiated, that of SHM and CSR, both of which are critically dependent on the enzyme AID(197). The mechanism of action of AID has created some controversy, however it is now generally accepted to act directly on single stranded DNA deaminating cytidine to uracil(198). An alternative mode of action, as an RNA editing enzyme, was originally proposed and suggested that as AID bears remarkable similarity to other RNA editing members of the APOBEC family (199), however overwhelming evidence now points to a direct deaminating action for AID.

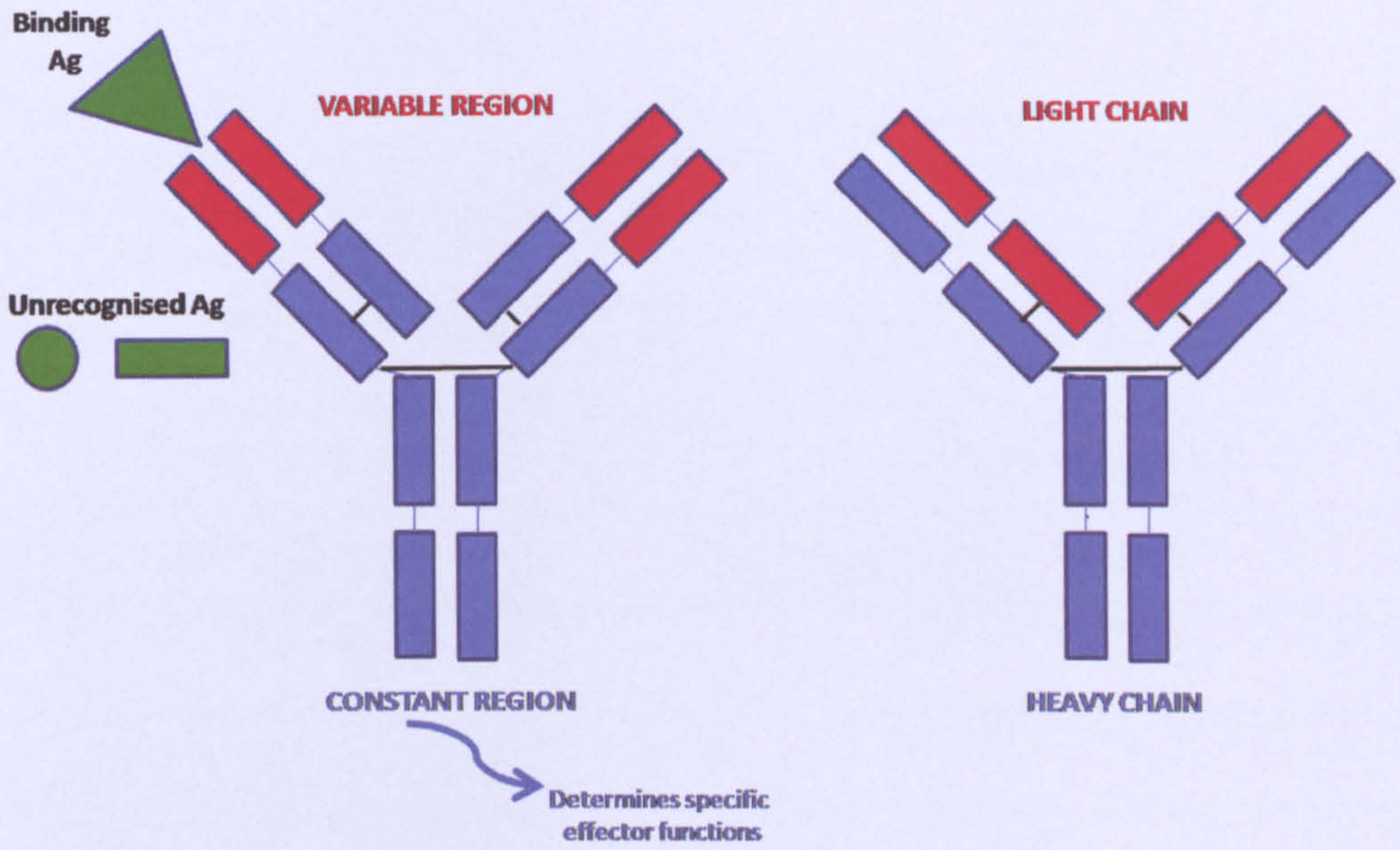


Figure 4: Basic structure of an antibody

Antibodies exhibit a characteristic Y-shaped structure composed of two identical heavy (shown in blue right of image) and light chain (shown in red right of image) polypeptides. The variable domains (shown in red on left of image) of both the heavy and light chains form the target binding region of antibodies, whereas the constant regions of antibodies (shown in blue on left of image) determine the specific immune effector functions of antibodies.

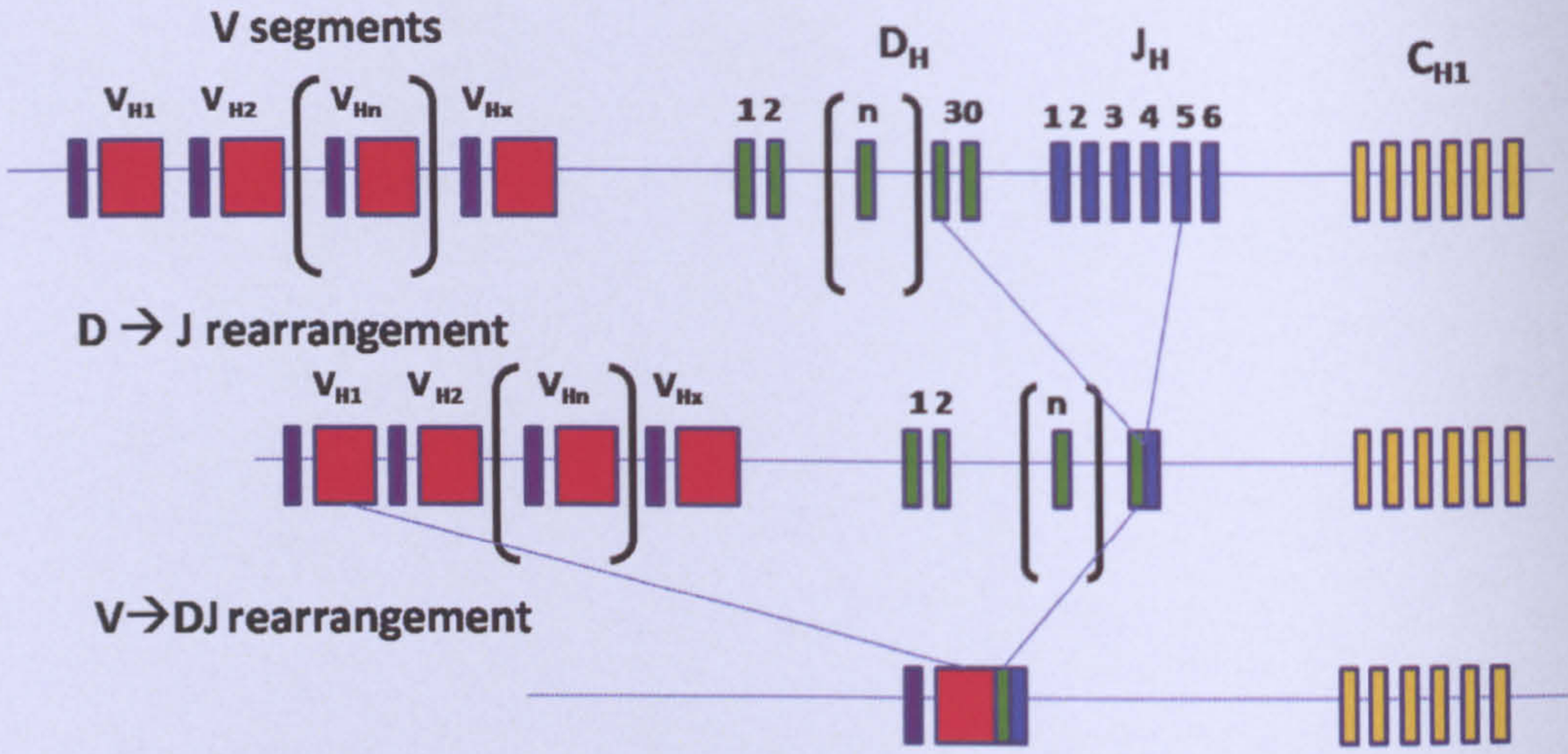

V segments
V_{H1} V_{H2} V_{Hn} V_{Hx}
D_H
1 2
n
30
J_H
1 2 3 4 5 6
C_{H1}
D → J rearrangement
V_{H1} V_{H2} V_{Hn} V_{Hx}
1 2
n
V→DJ rearrangement

Figure 5: VDJ rearrangement of the heavy chain immunoglobulin locus

Recombination of the variable (V), diversity (D) and joining (J) region segments of the immunoglobulin gene occurs early in B cell development within the bone marrow. V region family members number 50, D region members 30 and J region members 6. The process is under the control of RAG enzymes. The first step is a recombination of randomly chosen D and J segments, followed by recombination of the DJ segment with a randomly chosen V segment. This results in a mature transcript which is transcribed along with the constant region. Further variation is introduced by the action of terminal deoxynucleotidyl transferase, an enzyme that anneals the recombining segments and does so by the introduction of up to 15 new nucleotides. The intervening DNA that is not used is cut out and discarded.

Somatic hypermutation (SHM)

SHM is a process by which further variability is introduced into the VDJ region of the IgH chain locus via the introduction of point mutations. The action of AID to deaminate cytidine to uracil introduces mutations that are then processed following DNA replication as base excision repair or mismatch repair (Figure 6). SHM occurs within hotspots in the VDJ regions known as complementary determining regions (CDRs) (Figure 7), as the amino acids encoded by them have most contact with antigen and thus determine antigen binding affinity of antibody. Mutations occurring within the frame work regions determine folding of antibody and are thus selected against. SHM results in single base mutations in the vast majority of sequence changes with transition mutations exceeding transversions by up to 5:1. SHM is traditionally considered to occur solely within the environment of the GC where B cells expressing antibody with high affinity for retained antigen receive survival signals and those with low affinity undergo apoptosis. Thus repeated cycles of GC selection result in the selection of high affinity antibody. B cells exiting the GC have been demonstrated to display antibody with antigen binding affinity increased some 100 fold (reviewed in (200)) and are subsequently recruited immediately to the immune cascade or alternatively to the immune memory.

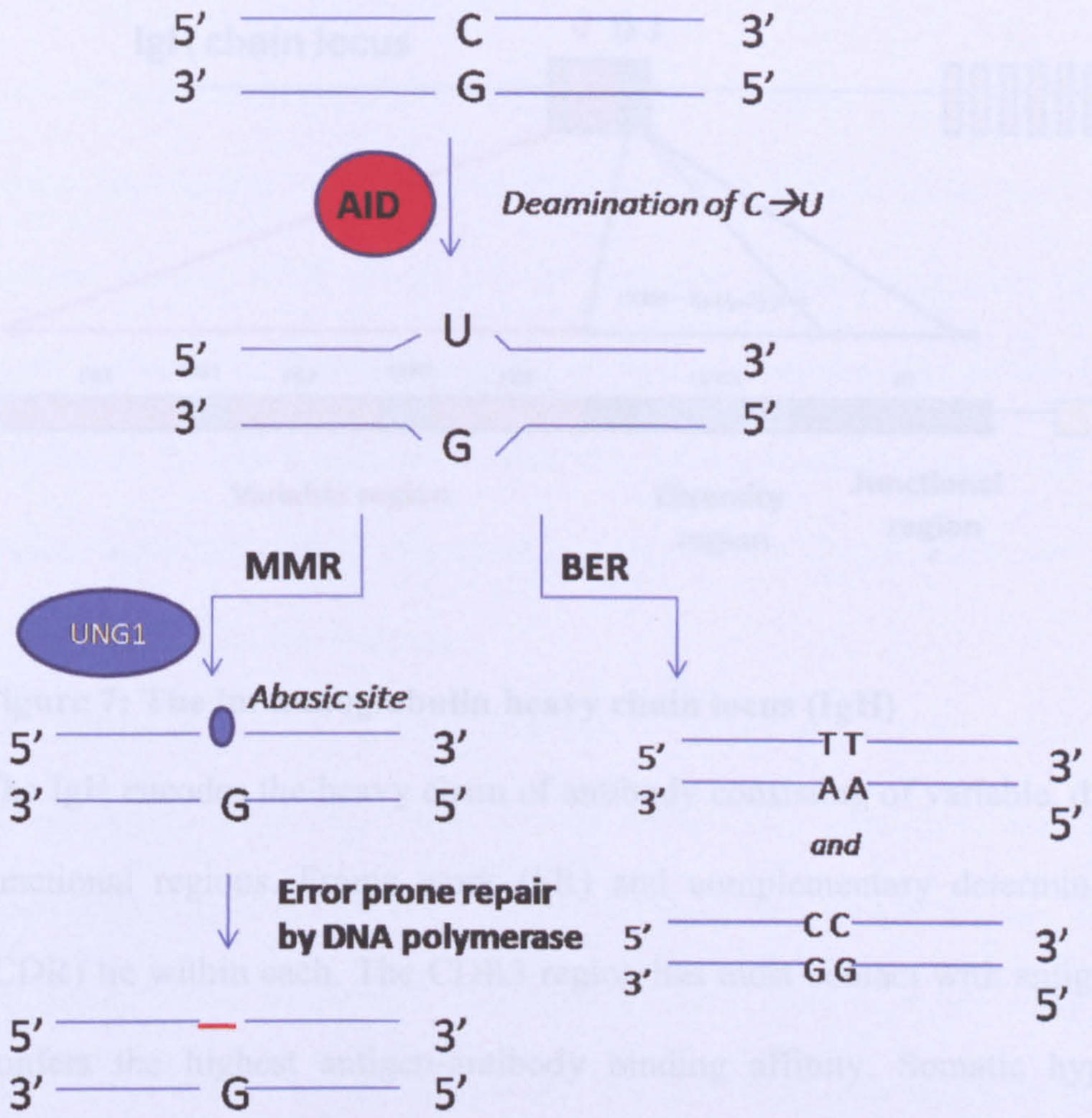


Figure 6: Mechanism by which activation induced cytidine deaminase mediates somatic hypermutation

Activation-induced cytidine deaminase (AID) deaminates cytidine residues in DNA, converting them into uridine. The U-G mismatch can then be processed either by base excision repair (BER) or mismatch repair (MMR). In somatic hypermutation, the mismatch can be replicated to produce a C-to-T mutation, nick processing by UNG can produce an abasic site, or a gap can be filled in by error prone polymerases.

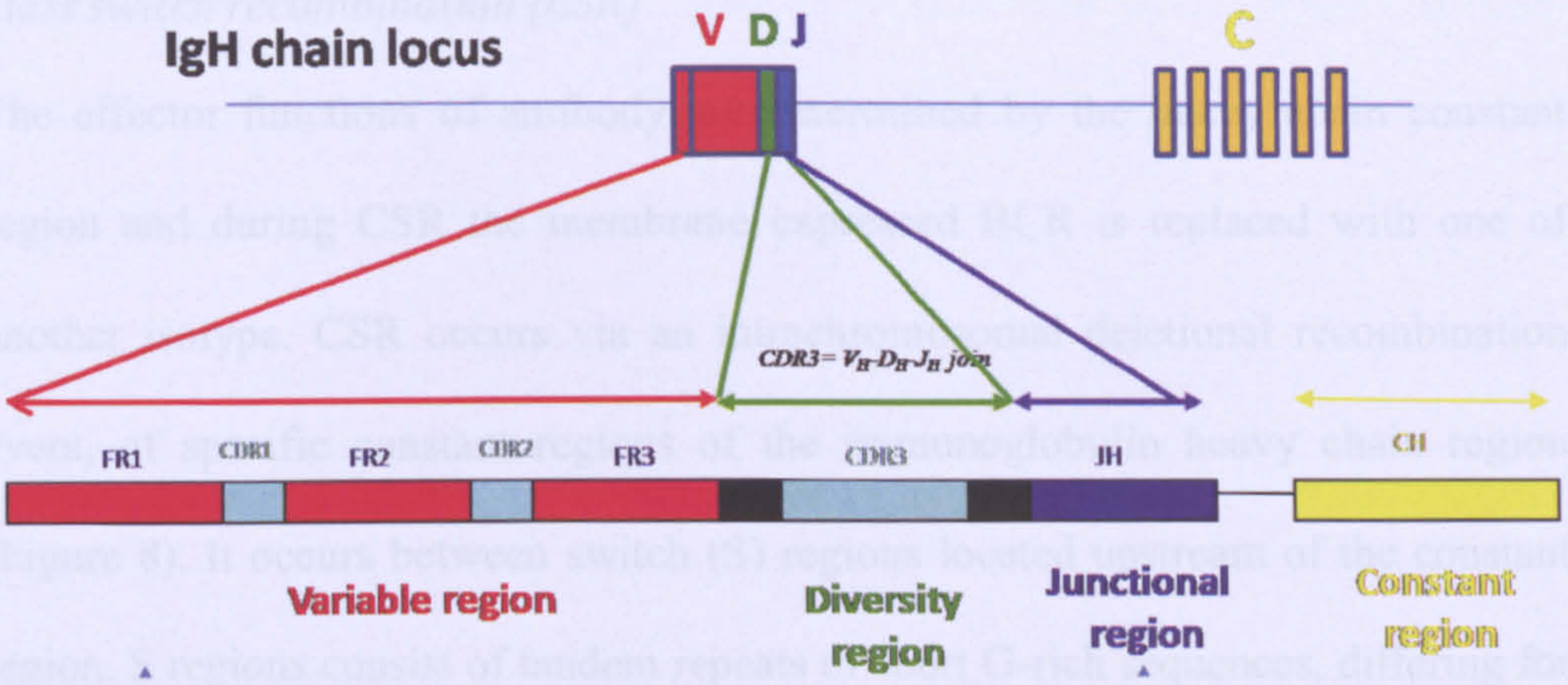


Figure 7: The immunoglobulin heavy chain locus (IgH)

The IgH encodes the heavy chain of antibody consisting of variable, diversity and junctional regions. Frame work (FR) and complementary determining regions (CDR) lie within each. The CDR3 region has most contact with antigen and thus confers the highest antigen-antibody binding affinity. Somatic hypermutation (SHM) preferentially occurs within CDR regions determining antigen binding, as oppose to FR regions that affect antibody folding and thus are selected against. A comparison of the CDR:FR mutation ratio allows determination of whether antigen driven SHM has taken place.

Class switch recombination (CSR)

The effector functions of antibody are determined by the heavy chain constant region and during CSR the membrane expressed BCR is replaced with one of another isotype. CSR occurs via an intrachromosomal deletional recombination event, of specific constant regions of the immunoglobulin heavy chain region (Figure 8). It occurs between switch (S) regions located upstream of the constant region. S regions consist of tandem repeats of short G-rich sequences, differing for each immunoglobulin isotype.

The first step in CSR is the production of germline (GL) transcripts specific for each S region. These GL transcripts are not actively transcribed but are thought to be necessary to direct AID to a specific S region and further to make the S region a suitable substrate for AID. CD40 ligation and/or TLR stimulation are required for the initiation of CSR with the specific isotype switch determined by local cytokine production by for by e.g. Th and DCs. AID then acts to introduce double stranded breaks within the S regions, resulting in subsequent looping out of intervening DNA and the product of a mature IgH locus. Repair occurs through a non homologous end joining repair system. A by product of this process is the looped out intervening DNA known as switch circles which contain a specific active I promoter, that directs the production of I-Cμ circle transcripts, that have been demonstrated to be a very specific and sensitive marker of ongoing CSR(201).

Extrafollicular induction of AID

Conventionally induction of AID expression and production of antigen specific antibody was thought to occur primarily within the microenvironment of the GC. Although IHC studies have recently led to the recognition that IF large B cells are capable of AID expression outside this environment. In addition recent data have

demonstrated that induction of AID expression can occur within B cells extrafollicularly within the gut and the spleen and that this expression is associated with CSR and low level SHM(202-204).

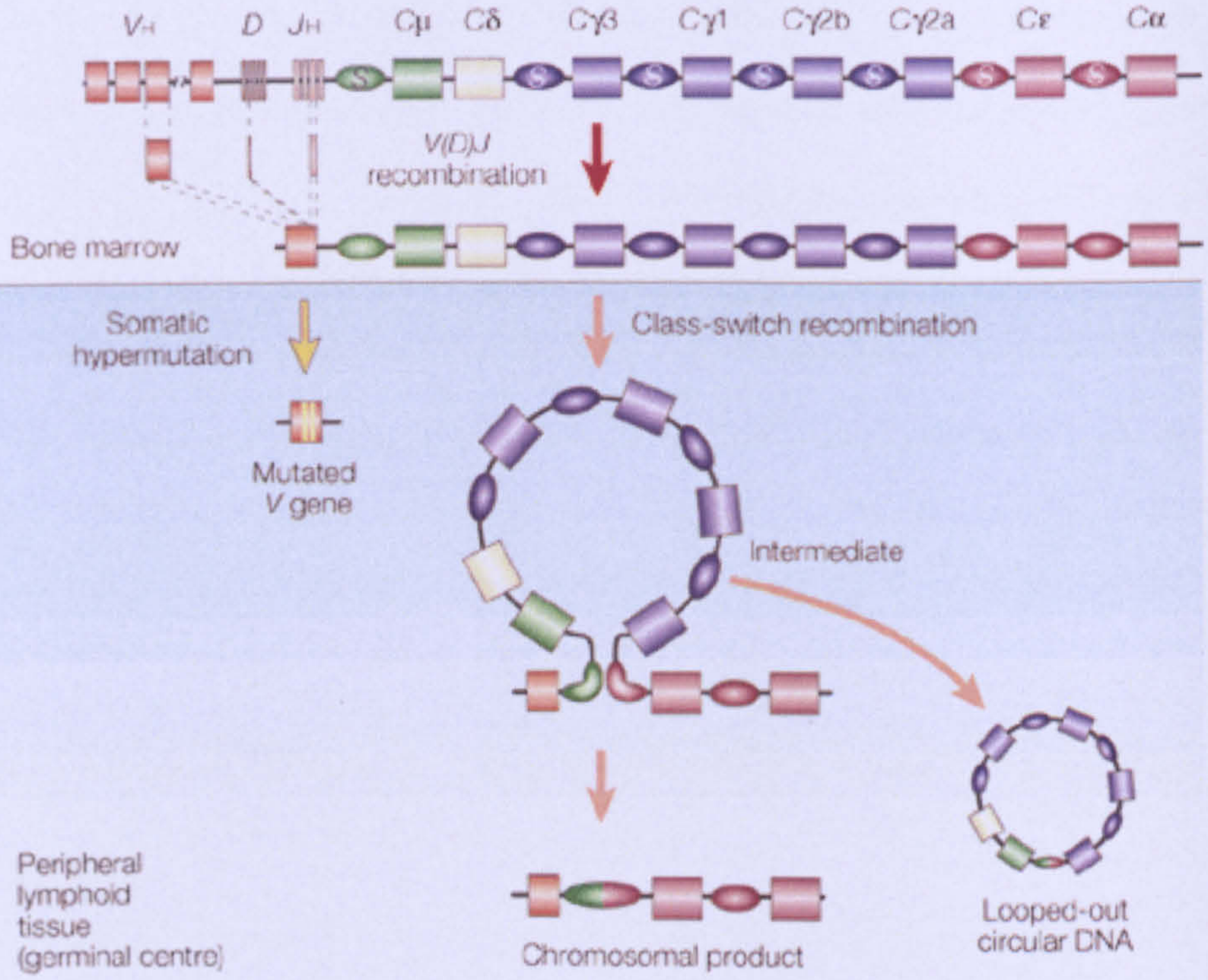


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Figure 8: Mechanism of class switch recombination

The mouse immunoglobulin heavy-chain locus is shown. Rectangles and ovals represent exons and switch (*S*) regions, respectively. *V*(*D*)*J* recombination occurs in the bone marrow, whereas somatic hypermutation and class-switch recombination occur in the peripheral lymphoid tissues. *C*lass-switch recombination brings the downstream constant (*C*) region exon in the proximity of the *V* exon by deletion between *S*μ and another *S* region upstream of the target *C* region. Deleted DNA is released as a circular DNA which is subsequently transcribed as a circle transcript. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cell Biology] ((1)), copyright(2001)

Antibody Isotypes

In mammals there are 5 classes of antibody each with specific effector functions (Table 3). Antibodies have three main functions, firstly they are capable of activating effector cells, they do this via binding initially to the invading pathogen and causing linking of sequential antigens, a number of cells are then stimulated via recognition of the Fc (constant region of antibody). Effector cells that recognise Fc receptors include neutrophils, phagocytes and natural killer cells, all of which by varying mechanisms are capable of destroying the invading pathogen. In addition antibodies are capable of activating complement following binding to pathogens and thus via the activation of the classical complement system bacteria are killed via opsonisation (ingestion by phagocytes) and/or the formation of a membrane attack complex, that allows direct antibody mediated killing.

Table 3: Antibody classes

Name	No of Subclasses	function
IgA	2	Located in mucosal areas (e.g. intestine, respiratory tract) and in secretions (e.g. tears, milk). Prevents pathogenic colonisation. Secreted in a dimeric form
IgD	1	The BCR on naive B cells yet to be exposed to antigen
IgM	1	Expressed on the surface of B cells and in a secreted form. Acts as part of the early humoral immune response. Secreted in a pentameric form
IgG	4	Provides the predominant antibody based defence against invading pathogens. It is also passed across the placenta, conferring passive immunity to the foetus.
IgE	1	It binds to allergens and triggers the release of histamine from mast cells and basophils, and further provides protection from parasitic worms

Mechanisms of inflammation

Cytokines

The role of the cytokine cascade in RA pathogenesis has perhaps received the most intensive research interest of that of any other inflammatory disease(205). Cytokines are small (5 to 50kDa) non structural proteins or glycoproteins that serve as chemical messengers between cells. This messaging system is of critical importance to both the development and functioning of the innate and adaptive immune systems.

Numerous proinflammatory cytokines have been demonstrated to be upregulated in both the sera and synovial fluid of patients with RA and the modulation of these systems has proven a powerful therapeutic tool.

The TNF superfamily

TNFα was identified in 1975(206). It is a soluble secreted 17KDa protein that exists as a trimer and binds to 2 different receptors TNFI and TNFII. Both receptors have intracellular regions and transducer signals for intracellular activation. Cleavage of the extracellular portion of the TNFα receptor results in the production of circulating soluble TNFα receptors, which are capable of sequestering TNFα and thus preventing binding to membrane bound receptors capable of cellular activation(207).

Following its identification, its capacity to degrade cartilage (208) and bone (209)in vitro raised the notion of a potential role for it in RA pathogenesis. Subsequent work demonstrating that blockade of TNFα in synovial mononuclear cell cultures resulted in the down regulation of IL1 (210)and GM-CSF(211), led to the proposal of a cytokine hierarchy controlling the inflammatory cascade. Further TNFα and its receptors were also shown to be expressed within synovial

tissue(212;213). These findings along with data showing that TNF transgenic mice develop a spontaneous arthritis (214)and that CIA could be ameliorated with the administration of a murine specific monoclonal antibody against TNFα(215) provided the rationale behind the first trials of TNFα blockade in RA patients.

A further member of the TNF superfamily, Lymphotoxin (LT) β has also been associated with RA pathogenesis. LTβ is a cell associated molecule and is known to be crucial for both the induction and maintenance of GC in secondary lymphoid organs(216), and has been shown to be differentially expressed within RA synovium containing these structures(217). LTα1β2 and Ligand for herpes virus entry mediator (LIGHT) are both ligands of the receptor for LTβ and have been implicated in RA disease progression(218). Therefore the blockade of these two ligands with a LTβR fusion protein is being explored as a possible therapeutic option in RA, although initial reports are not promising (reviewed in(205)).

Finally RANK-L a cytokine shown to be upregulated within the RA synovial membrane is known to be involved in RA pathogenesis. RANKL is expressed by fibroblast like synoviocytes and activated synovial T cells and has been shown to play a crucial role in osteoclast differentiation, maturation and activation and hence the associated bone damage characteristic of RA(reviewed in (219)). Expression of RANKL is itself regulated by TNFα as well as its naturally occurring inhibitor that of osteoprotegerin (OPG). The inhibition of RANKL is currently undergoing clinical trials in RA, with reports of efficacy in prevention of erosive damage(220).

The BAFF/APRIL pathway

The B cell activating Factor, BAFF (also known as TALL-1, BLyS, THANK and zTNF4) and a proliferation inducing Ligand, APRIL are both members of the TNF

superfamily(221;222). BAFF is a type II transmembrane protein, but can also be secreted following proteolytic digestion as a soluble polymeric or homotrimeric form(223). APRIL however is cleaved in the golgi before release and only exists as a soluble secreted form(224). In addition a splice variant of BAFF, ΔBAFF has been detected, that is not released from the membrane and is capable of acting as a negative regulator of BAFF, by interacting with the full length protein to form multimers(225;226). Finally APRIL is also capable of forming heterotrimers with BAFF (227)and a biologically active fusion protein with another member of the TNF superfamily, TWEAK (228).

BAFF and APRIL are expressed by monocytes, macrophages, DCs, T cells and FDCs(194;229-231). It has recently been demonstrated that non lymphoid cell lines are capable of expressing BAFF, and importantly for RA pathogenesis has included production by fibroblast like synoviocytes(232) and osteoclasts, the latter of which are also capable of expressing APRIL(233). The production of BAFF and APRIL by neutrophils, macrophages and DCs can be stimulated by a number of cytokines, in particular IFNγ, IFNα, IL10(229) and CD40 as well as lipopolysaccharides (LPS)(234) and peptidogylcans(235).

Three receptors for BAFF have been identified, BAFF-R (BAFF-receptor also known as BR3), TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor) and BCMA (B cell maturation antigen) and all are expressed on B cells(194). BCMA is highly expressed on plasmablasts, plasma cells and tonsillar GC B cells(236;237). BAFF-R is expressed by all peripheral B cells, although down regulated in GC B cells(236;237). TACI and BAFF-R are additionally expressed on activated T cells and BAFF-R on regulatory T

cells(238). APRIL binds with high affinity to just BCMA and TACI (194;239) and is also capable of binding to proteoglycans(238) (Figure **9**).

BAFF is known to be a crucial B cell maturation factor and this is illustrated by the lack of maturation of B cells beyond the immature T1 stage in mice deficient in BAFF(240-242) and further the overexpansion of the T2 and MZ B cell compartments in mice over expressing BAFF(243;244). In contrast, the role of APRIL is less well defined as conflicting results have emerged from two studies using APRIL knock out mouse models, one demonstrating no overt phenotype (245)and the other impaired class switching function, larger GCs and increased numbers of effector T cells(246).

From studies investigating the phenotype of both BAFF-R and BCMA deficient mice, it would appear that BAFF-R is the critical receptor mediating B cell survival as BAFF-R deficient mice appear similar to BAFF null mice(247) and BCMA deficient mice have only minor defects in the immune system(248). However the study of TACI deficient mice has demonstrated a negative role for this receptor in B cell activation, and as such survival mice have an over expanded B cell compartment(249-251). Such an inhibitory role may not be replicated in humans as mutations in TACI have been associated with immunodeficiency(252).

Of crucial interest to RA pathogenesis is work surrounding the role of BAFF in regulating B cell tolerance. The elimination of autoreactive B cells from the B cell compartment before they become long lived plasma cells appears to be dependent on the inability of these cells to respond fully to BAFF as well as controlled levels of BAFF expression (253-255) and indeed mice over expressing BAFF produce autoantibodies as well as developing other autoimmune manifestations(256;257) .

Elevated levels of APRIL and BAFF and BAFF/APRIL heterotrimers have subsequently been found in the sera of RA patients and moreover levels of APRIL correlated with levels of anti-glucose 6 phosphate isomerase (G6PI) (258) and levels of BAFF with RF(259). Further unique polymorphisms within the BAFF gene that are capable of regulating BAFF expression have been associated with RA, providing further evidence supporting a role for BAFF dysregulation in RA pathogenesis (260). Inhibiting BAFF via the use of TACI-Ig has generated promising preliminary data in the CIA mouse model(261) and in humans, where treatment was associated with clinical response and falling levels of both RF and anti-CCP antibodies(262). In addition clinical trials of the anti-BAFF monoclonal antibody belimumab have shown positive results in the treatment of SLE(263) and reports of ongoing clinical trials in RA are currently awaited. However, there are a number of hypothetical reasons for using anti-BAFF agents as adjunctive to anti-CD20 directed B cell therapy. Firstly BAFF is expressed on a slightly different variety of cells than CD20, for example plasma cells. Secondly the peripheral B cell depletion following treatment with anti-CD20 agents is followed by increased levels of peripheral BAFF in the sera of patients(264), which has the potential to provide survival signals to emerging autoreactive B cells, resulting in a subsequent resurgence in autoimmunity.

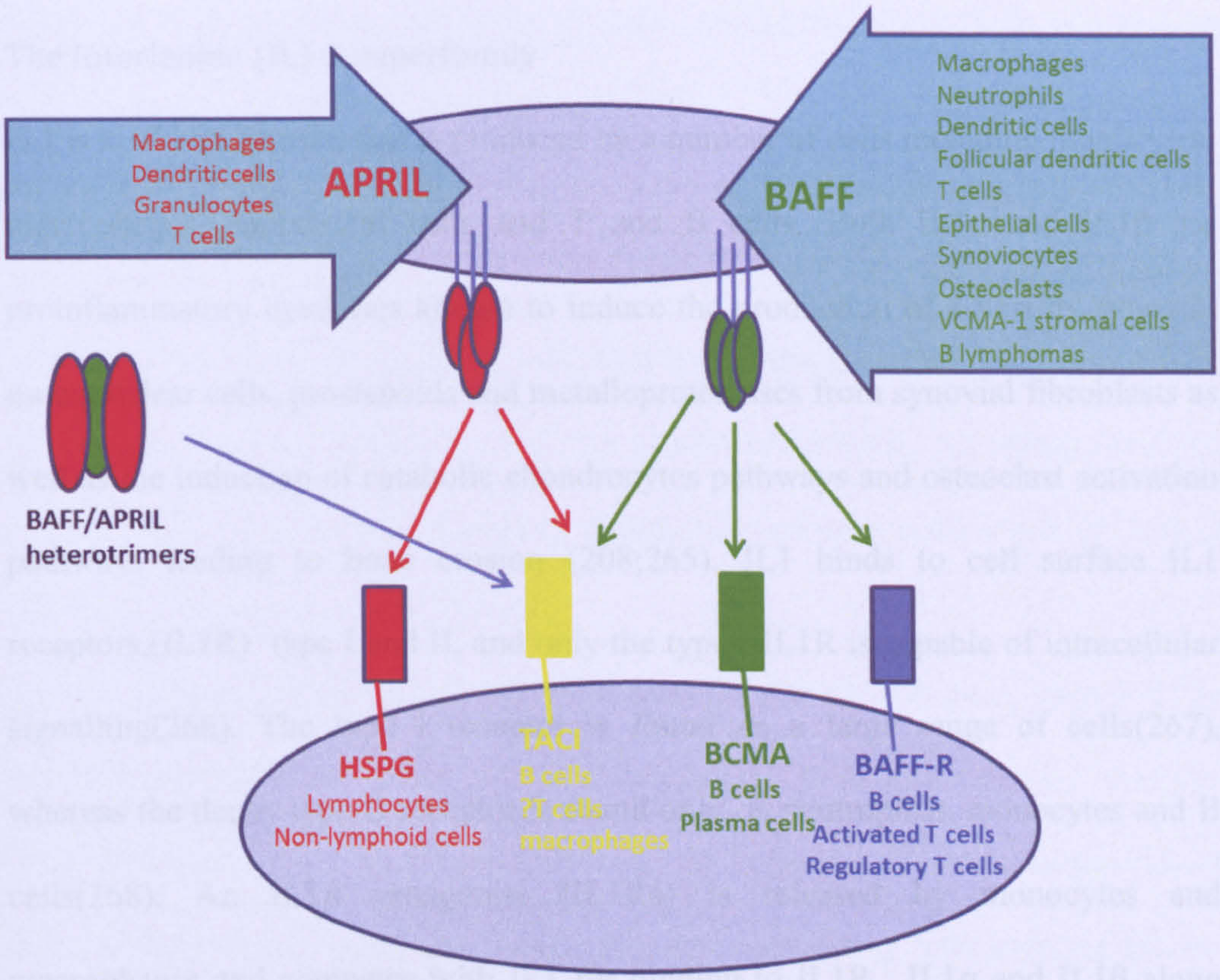


Figure 9: Interaction and expression of ligands and receptors in the BAFF/APRIL system.

A simplified version of the BAFF/APRIL pathway. Cell types that produce BAFF or APRIL are indicated in light blue boxes at the top of the figure with an arrow. In the bottom part of the figure receptors are represented left to right including heparin sulphate proteoglycans (HSPG). Below each receptor is a box that indicates cell types expressing these receptors. Interactions between ligands and receptors are shown by arrows (adapted from (2))

The Interleukin (IL)-1 superfamily

IL1 is a 17 kDa protein that is produced by a number of cells including monocytes, macrophages, endothelial cells and T and B cells. Both IL1α and IL1β are proinflammatory cytokines known to induce the production of TNFα by synovial mononuclear cells, prostanoids and metalloproteinases from synovial fibroblasts as well as the induction of catabolic chondrocytes pathways and osteoclast activation pathways leading to bone erosion (208;265). IL1 binds to cell surface IL1 receptors,(IL1R) type I and II, and only the type I IL1R is capable of intracellular signalling(266). The type I receptor is found on a large range of cells(267), whereas the decoy type II receptor is found only on neutrophils, monocytes and B cells(268). An IL1R antagonist (IL1Ra) is released by monocytes and macrophages and competes with IL1 for binding to IL1R. IL1α and IL1β along with IL1Ra have been found to be expressed by cells of the RA synovial membrane(269;270)

Animal studies have strongly implicated IL1 pathways in the pathogenesis of arthritis, with data demonstrating that intra articular IL1 injection results in cartilage degradation in rabbits(271) and the amelioration of CIA with the administration of monoclonal antibodies against IL1 in mice(272). In addition mice deficient in ILRa develop a spontaneous arthritis(273), but the transfer of IL1 blockade to RA patients, using a human ILRa has not met with such convincing results (274), although newer approaches e.g. monoclonal antibodies against IL1 are currently undergoing clinical trials (reviewed in (205)).

IL18, a further member of the IL1 superfamily has also been detected in RA synovium (275). Animal experiments have also implicated it in the pathogenesis of RA, with ameliorated CIA detected in IL18 deficient mice(276) as well as IL18

blockade reducing disease severity in CIA mice(277). Thus IL18 blockade is currently undergoing phase I clinical trials(278)

An IL18 inducible protein, IL32, has also recently been suggested to play a role in RA. A splice variant IL32γ has been demonstrated to be produced by synovial macrophages and promote PGE_2 and cytokine synthesis in vitro(279). IL32 has further been demonstrated in the CIA model of arthritis to induce articular inflammation in a TNFα dependent manner(280) and in patients levels of IL32 in the sera to associate with ESR (279).

Finally a newly identified family member, IL33(281), has been shown to be expressed within the RA synovial membrane, predominantly by synovial fibroblasts(282). It has been shown to be capable when administered of exacerbating CIA in mice and when deficient associating with reduced disease severity(282).

IL6

IL6 is an inflammatory cytokine produced by macrophages, monocytes, synovial fibroblasts and T cells. It has diverse biological functions including a role in B cell maturation to plasma cells, activation of cytotoxic T cells, the formation of osteoclasts, broad effects on haematopoiesis in the bone marrow and the proliferation of synovial fibroblasts (reviewed in (283)). It is primarily responsible for the acute phase response seen in RA and is implicated in the anaemia of chronic disease. Levels of IL6 in RA patients have also been shown to correlate in the sera with disease activity(284) and in the synovial fluid with radiological damage(285). The release of IL6 is induced by both TNFα and IL1. The receptor for IL6 is heterodimeric consisting of gp130 and IL6R, and both receptors result in cellular activation and so targeting either has therapeutic potential(286). Following

work demonstrating a crucial role for IL6 in the pathogenesis of CIA (287) the targeting of IL6R in RA patients using a specific monoclonal antibody, tocilizumab, was commenced. This has now been approved by the European Medicines Agency in combination with methotrexate for use in RA, following convincing clinical trial data(288-290) (291).

The IL12 superfamily

IL12 is a heterodimeric cytokine consisting of a p40 and p35 subunit, it is released by antigen presenting cells, DCs and monocytes and macrophages. As IL12 is the main inducer of IFNγ (292)and thus the production of a Th1 mediated inflammatory response(293), a central role in RA pathogenesis was thought likely. This hypothesis was supported by animal experiments demonstrating an augmented CIA response when IL12 was administered to mice(294) and attenuated disease when IL12 was blocked(295). In humans, data further supported a role for IL12 in disease pathogenesis with correlations reported between levels of IL12 in the sera of RA patients and clinical parameters(296;297) however these results have created some debate in the literature(205). IL12 shares a component of its heterodimer, the p40 subunit, with a more recently recognised cytokine, IL23(298), and further both cytokines share a component of their heterodimeric receptor, IL12βR1(299). The observation that mice deficient in either IL12 and/or IL23 did not develop CIA, as opposed to mice lacking only IL12 developing severe disease, led to the hypothesis that the autoimmune actions of IL12 were in fact attributable to IL23(300). The primary function of IL23 has been demonstrated to induce proliferation of Th17 cells, a proinflammatory T cell capable of the production of a number of inflammatory cytokines associated with the pathogenesis of RA(173;301).

Lastly, IL27 has been recently identified as a cytokine of the IL12 superfamily, a heterodimeric cytokine with a subunit homologous to a subunit of IL12(302). Binding of IL27 to its receptor in mice results in the proliferation of naive T cells and promotes a Th1 response, whilst suppressing the production of pro inflammatory Th17 cells(303). A role in the pathogenesis of RA is supported by the suppression of CIA in prone mice by the administration of recombinant IL27(304), but this anti inflammatory effect maybe dependent on disease stage, as severity of experimental allergic encephalitis could be reduced by the administration of IL27 neutralizing antibodies, suggesting IL27 is capable of mediating both pro and anti inflammatory effects(205).

IL17

IL17 is the major cytokine produced by Th17 cells and has wide ranging proinflammatory effects. It induces HLA class I expression, the production of a number of proinflammatory cytokines and is osteoclastogenic (reviewed in (305)). A role for IL17 in animal models of arthritis has been suggested by a number of reports demonstrating that deficiencies in IL17, either through neutralizing antibodies or genetic knock out result in ameliorated disease(306-308). IL17 has also been detected within the synovial membrane and synovial fluid of patients with RA(309) and strategies to block IL17 are currently underway in RA(205).

4α-helix family

This family of cytokines binds to receptors that contain γ_c and most attention within the area of RA pathogenesis has focused on IL15, IL7 and IL21. IL15 is an IL2 like cytokine that is produced by T cells, monocytes, fibroblasts and endothelial cells. It can stimulate the generation of cytotoxic T cells as well as activate a wide range of immune cells. It has been detected within the synovial

fluid and membrane of patients with RA and can induce the release of TNFα , IL17 and IFNγ(310;311). IL15 blockade has been shown to reduce the severity of CIA(312) and preliminary data from human trials of IL15 neutralization in RA patients have shown promising results(313).

IL7 appears a likely candidate for involvement in RA pathogenesis as it is detected in the sera(314), synovial membrane(315) and synovial fluid(316) of RA patients. It is also known to play a number of immune mediated roles including the induction of a proinflammatory response in synovial cell cultures(315;316) as well as playing a role in lymphoneogenesis(317), osteoclastogenesis (318)and modification of chrondrocyte function(319). In addition the persistence of IL7 expression post anti-TNF treatment in some patients(315;320), demonstrates a TNFα independent stimulatory pathway and suggests IL7 may well be an attractive option for therapeutic modulation.

Finally IL21 has been detected in the synovial fluid and synovial membrane from the joints of patients with RA(315;321;322). Blockade of IL21 in *in vitro* cultures of synovial cells has been shown to result in the down regulation of TNFα, IL1 and IL6(323). Further neutralization using an IL21 receptor fusion protein resulted in the amelioration of CIA in mice(324). It has additionally been shown to play a critical role in the differentiation of Th17 cells and thus these data together make IL21 an attractive pathogenic candidate for RA.

Anti-inflammatory cytokines in RA

Of note anti-inflammatory cytokines such as IL10(176) and cytokine-inducible suppressor of cytokines (SOCS)-1 and 3 (325)are also detectable in the inflamed synovial compartment. It is thought that levels of these cytokines are sub optimal for biological effectiveness and that this resulting disequilibrium between

proinflammatory and anti-inflammatory cytokines leads to inflammation and subsequent joint damage.

Chemokines (CKs)

CKs are low molecular weight (8-14Kd) structurally related, secreted or membrane bound proteins, that function as cell attractants. More than 50 human CKs have so far been characterised, along with 18 receptors (326;327).The binding of a CK on to its cognate receptor can induce a wide spectrum of effects, including the arrest and firm adhesion of blood-borne cells on endothelial surfaces and the transendothelial migration into tissues. CKs in particular allow leucocytes to circulate from the bloodstream to specific organs and move inside these tissues in a programmed fashion through spatial and temporal successions of CK gradients(328) but they can also participate in cell activation and are intimately involved in angiogenesis(329). CKs are produced in two main ways both constitutively and following up regulation in the context of inflammatory reactions.

Secondary lymphoid organs are among the best anatomical sites where the constitutive expression and role of CKs have been studied. Under homeostatic conditions T and B lymphocytes circulate from the blood stream to secondary lymphoid organs, but only a selected few (mainly naïve lymphocytes) are allowed to extravasate into the lymphoid tissues, via HEVs. This selectivity is due to the interaction between specific CK-Rs expressed by lymphoid homing cells and CKs constitutively expressed in secondary lymphoid organs for example the CK and CK-R pairings CCR7-CCL21 and CXCR5-CXCL13(330;331). Lymphoid CKs have a central role in both the formation and maintenance of embryonic lymphoid structures(332) directing specific leukocyte populations to discrete compartments

and favouring interactions between distinct cell populations (e.g. DCs and T cells(333)).

The main focus of interest in lymphoid CKs and RA arises from the relationship observed between chronic inflammation and ELN (217). Recent observations have demonstrated that the lymphoid neogenetic process is associated with the ectopic production of lymphoid-constitutive CKs, such as CXCL13 and CCL21, which are constitutively produced in the B and T cell rich areas of secondary lymphoid organs, respectively(334;335). Importantly, lymphoid CKs have been shown to be powerful lymphoid tissue morphogenetic factors capable, when over expressed ectopically in the pancreas of transgenic mice, to act upstream in the process of lymphoid neogenesis(336).

The expression of both CXCL13 and CCL21 have been shown to be critical to the process of ELN within the rheumatoid synovial membrane, with data demonstrating production in minor and unstructured aggregates (334) and further the demonstration by Weyand et al (217) that both these CKs (together with LTβ) can predict the presence of different levels of organizational structures in patients with RA.

Other CKs constitutively produced in secondary lymphoid tissues, such as CCL19 and CXCL12, have been shown to be induced or up regulated in RA synovium. CCL19 involved together with CCL21, in the recruitment and localisation of CCR7+ lymphocytes and mature DCs within secondary lymphoid tissue T cell areas, has been found to be expressed at high levels by dendritic cells of RA patients with active disease. In addition CCL19 mRNA has been found to be expressed at increased levels in synovial tissue from RA patients than those with OA(337).

The participation of CXCL12 and its receptor (CXCR4) in the pathogenesis of RA has been previously suggested by several studies that have reported an up regulation of CXCL12 in RA synovium and a role for the ligand/receptor system in local leukocyte attraction(338) and further by *in vivo* data demonstrating that when CXCL12/SDF-1 is injected into human synovial tissue grafts implanted into SCID mice, U937 monocytoid cell recruitment from the bloodstream is promoted(339). Further CXCL12/SDF-1 is a TNFα independent CK(340) and its persistence in the synovia of patients treated with anti-TNFα suggests that it may be involved in alternative pathways mediating synovitis in RA(341).

RA synovium has been shown to contain a complex mixture of other inflammatory-inducible CKs including CCL2(342), CCL3(343), CCL5(344), CXCL10(345), CXCL9(346), CXCL6(347) and CXCL5(348) functioning in the recruitment of effector cell population such as neutrophils and activated T cells.

In addition, CK and CK-Rs are also known to be involved in the migration of DCs, draining antigen from peripheral tissues, to secondary lymphoid organs where the priming and activation of naïve lymphocytes takes place and similar mechanisms of up and down regulation of CKs and CKRs are thought to act in recruiting antigen presenting cells such as immature DCs from the blood stream to the inflamed joint and in the migration of primed mature DCs to the local lymph node.

While, as mentioned above, CXCL12/SDF-1 appears to be produced independently from TNFα, the production of many inducible CKs is mostly regulated by inflammatory cytokines such as IL-1 and TNFα, known to play a critical role in synovial inflammation, via processes (amongst others) of

angiogenesis, integrin activation, chemotaxis and release of other mediators (e.g. MMPs).

CXCL8/IL-8 is one of the best studied inflammatory CKs. It is produced by macrophages in the synovial compartment(349) and by fibroblasts following stimulation with IL-1α, IL-1β, TNFα or LPS (350). CXCL8/IL-8 has been shown to induce synovial inflammation in animal models (351)and to be up regulated in RA, specifically in affected joints(352), with its levels in the sera of RA patients correlating with disease activity(349). Furthermore, CXCL8/IL-8 is a potent mediator of angiogenesis and this function that has been demonstrated also in the RA joint(353).

As mentioned, several other inflammatory CKs have been found to be up regulated in the serum, synovial tissue or synovial fluid of RA patients. However, given the nature of human studies most data elucidating the exact dynamics of inflammatory CK production and function has been established in animal models of arthritis. In rat adjuvant induced arthritis (AIA) higher levels of CCL3/MIP-1α were shown early in the course of the disease and higher levels of CCL2/MCP-1 predominantly in the later stages(354)

Other specific functions of CKs have been elucidated and they include the role of CXCL1/GROα in RA, which has recently been suggested as a potent stimulator for release of BAFF from neutrophils(355). Further, CXCL4/PF-4 is known to play a role in angiogenesis and has been suggested as a marker of RA associated vasculitis(356).

Although there are many animal studies demonstrating the possibilities of manipulation of the CK system as therapy in RA, there has only been one published clinical trial investigating the effect of CCR2 inhibition on RA and this

demonstrated a lack of effect on clinical response or synovial biomarkers(357). This lack of response, as well as little further published data, is potentially due to several limitations including; the difficulty in using the prophylactic approach in arthritis prevention in humans; the fact that many CKs and receptors are species specific and the possibility that some ligands may act as agonists at one receptor and antagonists at another.

The rheumatoid joint

Histopathology of the synovial membrane

The normal synovial membrane

The normal synovial membrane covers the inner surface of joints and is a relatively acellular structure approximately 0.5-5mm thick. It consists of a loosely organised intimal lining layer 1-2 cells deep and a thicker sublining connective tissue layer. The lining layer is composed of 2 cellular subtypes: 1) Type A the macrophage-like synoviocyte and, 2) type B the fibroblast-like synoviocyte(358;359). The sublining layer consists of a matrix of collagen, fibronectin, lamin and proteoglycan interspersed with macrophages, adiopocytes and type B fibroblasts(360). The synovium functions to produce synovial fluid through the production of hyaluronidan, lubricin and surface-active phospholipids, synthesised by both types of fibroblasts(361-363). Thus the synovial membrane and synovial fluid enables joint protection, lubrication and a facility by which nutrients can be transported to the joint cartilage and debris can be cleared(364).

The synovial membrane in rheumatoid arthritis

RA synovium, in comparison to that found in normal joints, has a transformed phenotype, with the ability to aggressively invade adjacent bone and cartilage.

Macroscopically the synovium appears thickened and hyperaemic with fronds of tissue within the joint. Microscopically the intimal lining layer is thickened to a depth of around 8 cells(365) and is associated with a cellular infiltrate of the sublining layer with pronounced angiogenesis. The predominant cellular infiltrate, accounting for up to 80% of cells of the sublining layer is type A fibroblasts which have been identified as infiltrating rather than proliferating cells originating from the bone marrow(366-368). This inflamed synovium is named pannus and at the interface with bone and cartilage multinucleated cells with an osteoclast phenotype appear and it is these that mediate the associated bone and cartilage destruction (Figure 10). Macrophages and synovial fibroblasts also release matrix metalloproteinases (MMPs), aggrecanases and serine proteases that digest collagen and proteoglycans degrading the extracellular matrix. This hyperplastic synovium is associated with an increased volume of synovial fluid with a hypercellular infiltrate consisting of largely polymorphonuclear cells, but also mononuclear cells, dendritic cells (DCs), T cells, B cells and natural killer cells as well as cellular and matrix debris(369-374).

The marked angiogenesis seen within the sublining layer is known to be a prominent feature of early synovitis and thus permits the transfer of oxygen, nutrients and inflammatory cells to the synovium. The stimulus for angiogenesis is thought to be increased metabolic demand and hypoxia. A range of growth factors, cytokines and chemokines are known to influence angiogenesis within the synovial membrane and in particular vascular growth endothelial factor (VGEF) has been demonstrated to be expressed within both synovial tissue and fluid from RA patients. Further fibroblast 1 and 2 growth factors, platelet derived growth factor and hepatocyte growth factor have all been found to be expressed within

synovial tissue (reviewed in (375;376)). The importance of angiogenesis in permitting synovial inflammation in RA has been further emphasised by the success of pro-angiogenic blockade in animal models of arthritis, for example with a soluble receptor for VEGF in the collagen induced arthritis (CIA) model(377). Finally the expression of angiogenic inhibitors in RA such as endostatin and thrombospondin 1 and 2 suggests that the hyperaemic synovium may well be a result of an imbalance between angiogenic promoters and inhibitors(378-380).

The cellular infiltrate in RA is composed of mononuclear cells, including $CD4^+$ and $CD8^+$ T cells, plasma cells, B cells, NK cells, monocytes, mast cells and DCs. The cellular infiltrate is recruited via the expression of a number of adhesion molecules on endothelial cells, including E-selectin, ICAM-1 and VCAM-1(381). The organisation of lymphocytes into aggregates, a process termed ectopic lymphoneogenesis (ELN) is well recognised in RA and is discussed in detail on page 84.

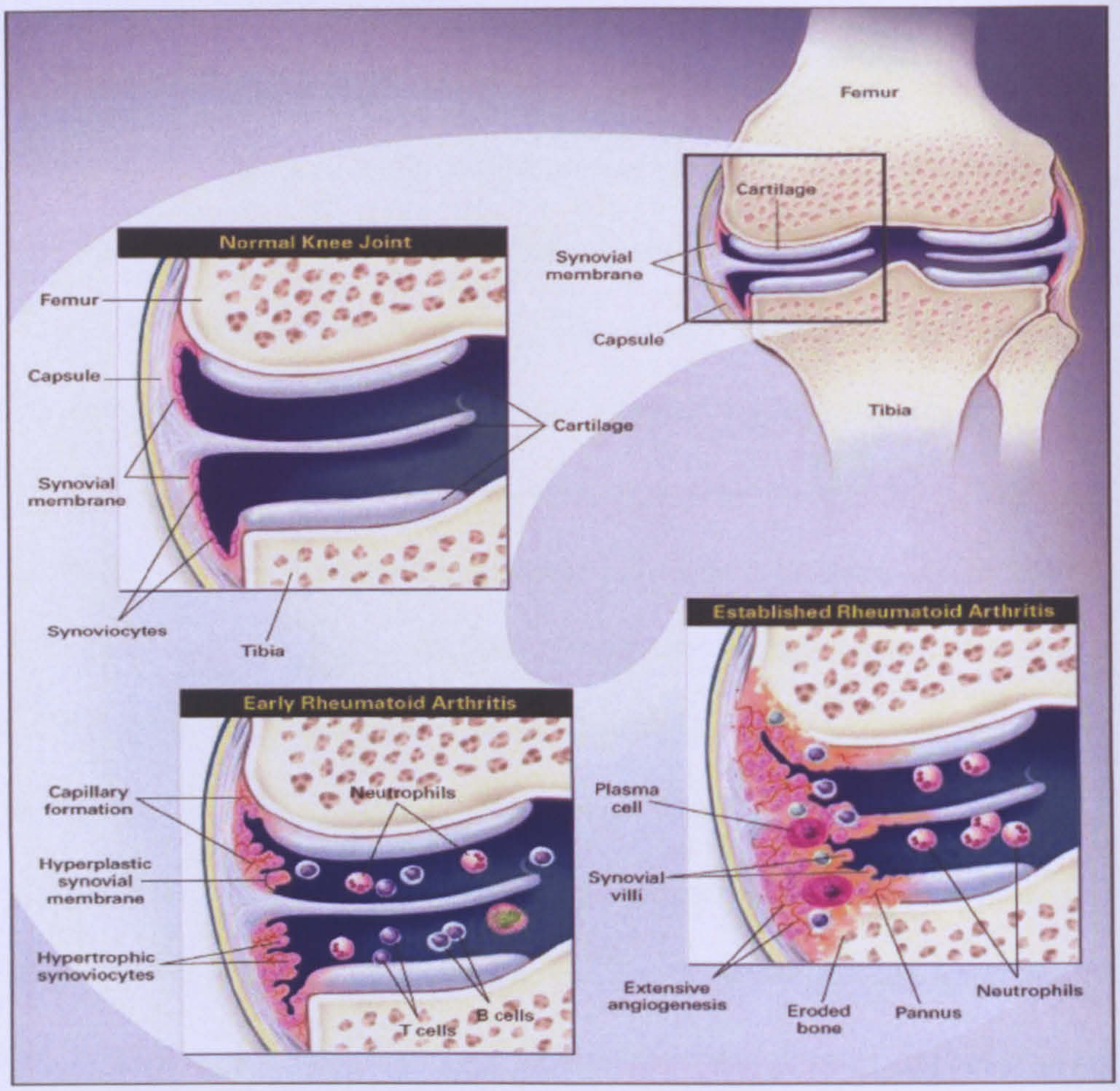

Femur
Cartilage
Synovial membrane
Capsule
Tibia
Normal Knee Joint
Femur
Capsule
Cartilage
Synovial membrane
Synoviocytes
Tibia
Early Rheumatoid Arthritis
Capillary formation
Neutrophils
Hyperplastic synovial membrane
Hypertrophic synoviocytes
T cells
B cells
Established Rheumatoid Arthritis
Plasma cell
Synovial villi
Extensive angiogenesis
Eroded bone
Pannus
Neutrophils

Figure 10: The synovial membrane in rheumatoid arthritis

In the normal knee joint, the synovium consists of a synovial membrane (usually one or two cells thick) and underlying loose connective tissue. Synovial-lining cells are designated type A (macrophage-like synoviocytes) or type B (fibroblast-like synoviocytes). In early rheumatoid arthritis, the synovial membrane becomes thickened because of hyperplasia and hypertrophy of the synovial-lining cells. An extensive network of new blood vessels is formed in the synovium. T cells (predominantly CD4+) and B cells (some of which become plasma cells) infiltrate the synovial membrane. These cells are also found in the synovial fluid, along with large numbers of neutrophils. In the early stages of rheumatoid arthritis, the synovial membrane begins to invade the cartilage. In established rheumatoid arthritis, the synovial membrane becomes transformed into inflammatory tissue, the pannus. This tissue invades and destroys adjacent cartilage and bone. The pannus consists of both type A and type B synoviocytes and plasma cells. Reproduced with permission from (382)

Ectopic lymphoneogenesis (ELN) in rheumatoid arthritis

In a number of autoimmune diseases, including Sjogren's syndrome, Hashimoto's thyroiditis and myasthenia gravis, as well as RA, the lymphocytic infiltrate of the target organ can become organised into GC-like structures(383). In RA the localization of T and B lymphocytes forming discrete perivascular clusters in the synovium is highly variable and can be the prominent picture in a subset of biopsies as well as in extrasynovial compartments such as the bone marrow (384;385) and the lung (386). Histomorphometric analysis adopted by the group of Professor Pitzalis and others based on the aggregate radial cell count has led to the development of a grading system (G1,2, and 3) that has been used to investigate the relationship between microanatomical variables, cellular-molecular pathways involved in lymphoid organization and their link with clinical outcomes(334;387-391). Using this system, recent studies in large cohorts of consecutive patients, analyzed at a single time point, have demonstrated a variable but consistent presence of synovial T–B lymphoid aggregates in approximately 30% (Grade 2–3, n = 103) (53) and 73% (Grade 2–3, n = 86) (387) of the specimens after arthroscopic biopsy in active knees, while they were detected in 44% (n = 64) of joint replacement samples in late stage arthritis(217). Although these data cannot be directly compared due to the different methods of analysis, previous studies performed in a single centre by the group of Bresnihan indicate the existence of a significant difference in the presence of lymphoid aggregates between bioptic specimens (36%, n = 29) and samples from joint replacement surgery (68%, n = 22) (392). These latter results suggest that these parameters may be influenced by the disease stage or by sampling procedures. It should also be noted that the characteristics of the inflammatory infiltrate in the joint might be influenced by the

historical period of sample collection. As effective treatment can modify synovial histology, the recent introduction of tight control and more aggressive regimens in early disease stages should be considered as an additional variable.

Whether this concept, proposed in a recent review article by Brennan and McInnes(205) can be applied to the prevalence of synovial lymphoid architecture in different cohorts remains to be proven. As the presence of lymphocyte clusters does not necessarily imply the organization of TLS, a critical issue is whether the process of lymphocyte aggregation in the synovium merely represents a disorganized accumulation of circulating cells or whether it can lead to a specific reprogramming phenomenon (lymphoid neogenesis) leading to the acquisition of structural and functional features constitutively involved in secondary lymphoid tissue immunological response (TLS organization).

The recognition however, that lymphoid neogenesis takes place within a proportion of ectopic lymphoid clusters in human diseases(383) has led to the hypothesis that the process may be controlled by reactivation of pathways physiologically involved in secondary lymphoid organ embryonic development and adult homeostasis(393). Among the factors involved in these processes, a central role is played by the lymphoid chemokine CXCL13 and the CCR7 ligands CCL21 and CCL19. These molecules, which are constitutively produced in the B- and T-cell rich areas of adult secondary lymphoid tissues, respectively, are

induced prenatally in subsets of VCAM-1+ICAM-1+LTbR+ embryonic mesenchymal cells (defined 'organizer cells') in the lymphoid anlagen and are essential for the local recruitment of CD3/CD4+IL-7R+RANK+ embryonic haemopoietic cells (defined 'inducer cells'). Inducers cells, whose survival, differentiation, and expansion is supported by other signals, including IL-7R

ligands and RANK-L, provide the pivotal source of the TNF family member LTβ which is in turn required for HEV differentiation, lymphoid chemokine expression amplification, and stromal architecture development (332) (Figure 11A).

Although the requirement of conventional 'inducer-organizer cell' interactions in the mechanisms of adult ectopic lymphoid neogenesis is debated(394) , the possible activation of similar molecular pathways in RA has been proven by gene expression studies demonstrating that CXCL13, CCL19 and CCL21 can be aberrantly up regulated in the synovium, in association with increased expression levels of LTβ, of genes related to the IL-7 signalling pathway, and that these systems are in turn linked with the presence of tertiary lymphoid structures (TLS) (217;317). Functional evidence of the ectopic morphogenetic potential of CXCL13, CCL21, and CCL19 is provided by over expression experiments in normal mice demonstrating that these factors are sufficient to promote downstream organization of lymphoid tissue in the pancreas (336;395) and thyroid (394). This process, whose developmental steps have been dissected in neo-forming clusters of progressively increasing size in CXCL13 transgenic animals(336), has been proven to be dependent on LTβ delivered by incoming B lymphocytes and by the number of clusterising cells. In keeping with these findings, by examining through a grading system of lymphoid aggregate size, the inflammation-induced process in humans, the synovial environment has been demonstrated to be able to acquire specific lymphoid-like features, such as the formation of high endothelial venules (HEV), the enrichment of T and B cells in partially separated areas, the differentiation of follicular DC (FDC) networks and the local synthesis of homeostatic chemokines, and that these processes are linked with the formation of ectopic lymphoid tissue and with the local enrichment of B cells(334). As

lymphoid neogenesis is invariably activated in aggregate tissues and most of the synovial clusters lack complex lymphoid structural features (334), the same studies also demonstrated the relationship between chemokine expression and different degrees of lymphoid arrangement *in situ*. This work demonstrated that in an ectopic setting, CXCL13 and CCL21 can be synthesized both in the context of highly organized structures, with a trend to segregate with FDC, B and T cells, as well as in minor organizational stages, devoid of FDC networks and with intermixed lymphocyte distribution(334). Thus, under inflammatory conditions in humans, reprogramming of lymphoid molecular pathways can associate with reprogramming of cellular and structural features reminiscent of conventional lymphoid organs while lymphoid chemokine expression can be regulated independently from and possibly upstream of TLS organization, as has been shown during murine embryonic development and in experimental systems. Confirming the possibility that ectopic CXCL13 production can be independent of GC-localized FDC (considered the main source of this factor inside synovial lymphoid aggregates), it has been recognized that different subsets of haemopoietic cells such as monocyte-macrophages (396) and antigen-experienced CD4+ T lymphocytes(397) are capable of expressing CXCL13 in the joint, emphasising that both the innate and adaptive immune systems could play a critical role of in the organization of TLS. These data, together with the recognized absence of primary follicles in the synovial environment(217) (55, 62), raises the possibility that the organizational process inside synovial aggregates, rather than being exclusively controlled by infiltration and aggregation-driven events, might be also dynamically influenced by the local immunological environment, as is the case with inducible lymphoid tissues(398) Figure 11B).

Vascular remodelling in synovial lymphoid aggregates

Beside their role in lymphoid tissue structural organization, lymphoid chemokines are also instrumental in regulating constitutive trafficking of naive and central memory T–B lymphocytes, a fundamental functional platform for homeostatic immune surveillance. Mechanistically, lymphocyte homing to LN is mainly sustained by the sequential binding of naive lymphocyte expressed L-selectin to peripheral node addressins (PNAd) on HEV, followed by firm adhesion and transmigration promoted by the activation of the lymphocyte chemokine receptor CCR7 by its locally expressed ligands CCL21 and CCL19. The same factors have been shown to be instrumental in the recruitment of CCR7+ mature DCs from afferent lymphatics, in their intra-tissue positioning in the T-cell-rich area, and in the establishment of the T–DC immunological synapse(399). Although this molecular apparatus is normally absent outside lymphoid tissues, several studies have demonstrated that both in RA and spondyloarthropathies, the inflamed synovial vasculature can undergo molecular remodelling with the acquisition of features typical of peripheral LN, such as high endothelial morphology and luminal expression of PNAd (334;387;400-402). These data have been confirmed by subsequent reports showing co-localization of synovial PNAd with the HEV-restricted sulfotransferase GlcNAc6ST-2 (also known as HEC-GlcNAc6ST, GST-3, LSST, or CHST4), an essential enzyme for the elaboration of L-selectin functional ligands as well as a critical epitope recognized by theMECA-79 monoclonal antibody(403).

To investigate further whether the molecular requirements for homeostatic lymphocyte trafficking can be re-programmed in the synovium, the characteristics of synovial ectopic HEV in relationship with chemokine expression have recently

been analyzed(50). These studies confirmed that a proportion of synovial HEV inside lymphoid aggregates can co-express PNAd in association with periendothelial synthesized CCL21, a distribution characteristic of human secondary lymphoid tissues (334;396). Intraaggregate expression of CCL21, detected in about 40% of Grade 3 synovial clusters and in the majority of the samples with Grade 3 lymphoid aggregates, was mainly found to co-localize with smooth muscle actin (SMA)+ CD45) myofibroblast-like stromal cells(404), a phenotype that has been demonstrated in independent studies to identify the cellular source of CCL21 in human and murine LNs(404;405). Collectively, these studies demonstrate the potential of the synovial stroma to re-program and acquire features of the vascular homing apparatus involved in lymphocyte homeostatic trafficking both at the morphological, cellular and molecular level, and that a vestige of the T-cell area of secondary lymphoid organs can form at ectopic sites.

DC-T-cell immunological synapse

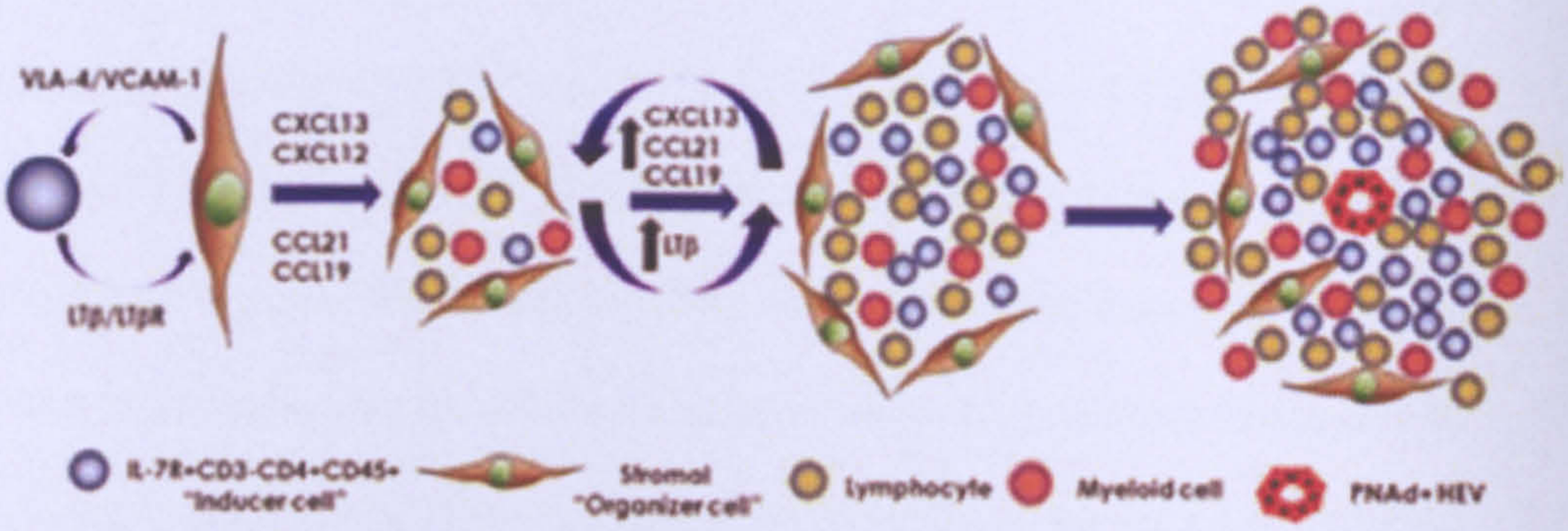
Despite the above discussed similarities, synovial lymphoid aggregates can show appreciable differences from secondary lymphoid organs including the frequent absence of a complete T–B-cell segregation (often cells are interspersed) and a significantly lower number of SMA+ CCL21-expressing cells, with expression often limited to occasional cells within poorly demarcated T-cell-rich areas(334;404;406). Whether these qualitative and quantitative differences determine an altered functionality in terms of cellular recruitment and immunological activation is unclear. Previous studies from Miossec's group (407) demonstrate that despite the well-known enrichment of immature DCs (typically found in inflamed tissues) in the synovium compared to secondary lymphoid tissues, CCR7+DC-LAMP+ mature DCs are positioned specifically within

lymphoid aggregates co-localizing with intra-aggregate expressed CCR7 ligands. These data, confirmed in a further independent cohort (404), suggest that synovial lymphoid aggregates might actually acquire the potentiality to retain mature DCs through the establishment of chemokine gradients, creating a favourable environment for local antigen presentation. Additional evidence has been provided by Baeten et al.(408), who demonstrated that despite the increased alterations in T-cell receptor CDR3 length distribution associated with diffuse synovitis, the HLA DR4-complexed cartilage autoantigens gp-39 is presented in the synovium by subsets of DC co-localizing with ectopic lymphoid aggregates (409). Although direct proof that T-APC immunological synapses are established within the synovium and in synovial follicles is missing, recent transcriptional screenings of RA synovium demonstrated a relationship between the presence of TLS and gene signatures related to T-cell receptor signalling and co-stimulatory pathways involved in T-cell activation (317). Whether naive lymphocyte priming(402) can contribute to these molecular traits or whether the synovial environment represents a 'privileged' niche for re-activation of antigen-experienced T cells (the main component of lymphoid aggregates)(410) and amplification of secondary responses, is unknown.

Class-switch recombination and somatic mutation: role of activation-induced cytidine deaminase

Physiologically, antigen-driven antibody responses take place within GCs of secondary lymphoid tissues where the processes of somatic hyper mutation (SHM) and class-switch recombination (CSR) of the Ig genes in GC-B cells occur which lead to affinity maturation and differentiation to memory B and plasma cells (81, 82). It has now been clarified that both SHM and CSR are initiated by and

critically dependent on the expression of the enzyme AID (197). Although the exact mechanism of action of AID has not been fully elucidated, AID initiates SHM by introducing single point mutations in WRC (W = A/T, R = A/G) motifs hot spots within the Ig variable genes, which encode for the antigen-binding region of the antibody (199;411). This process results in affinity maturation occurring through cycles of antigen-dependent selection within the GC. In addition, AID regulates CSR via its carboxyterminal region(412) by inducing double-strand breaks and recombination of Ig switch regions of DNA followed by excision of switch circles within the constant heavy-chain region. Ig class switching from IgM and IgD to IgG, IgE, or IgA profoundly influences antibody biological functions dramatically enhancing their effector capacity(413). In the mid-90s, several groups - by performing analysis of the V-gene repertoire from total RA synovial RNA extracts or from follicular structures microdissected from RA synovium - provided clear demonstration that a significant proportion of B lymphocytes accumulating within the synovial membrane of RA patients display an oligoclonal repertoire with highly mutated V regions, compatible with a local antigen-driven GC like reaction(414;415). These data provided strong indirect evidence that RA synovium can support *in situ* diversification and expansion of B-cell clones. However, the definite proof that synovial lymphoid follicles can actively sustain the molecular machinery responsible for affinity maturation and class-switching has been lacking and has been challenged by recent analyses in whole tissue extracts reporting the lack of direct relationship between lymphoid aggregates, B-cell clonal expansion and Ig somatic hypermutation(416).


VLA-4/VCAM-1
LTβ/LTβR
CXCL13
CXCL12
CCL21
CCL19
CXCL13
CCL21
CCL19
LTβ
IL-7R+CD3-CD4+CD45+
"Inducer cell"
Stromal
"Organizer cell"
Lymphocyte
Myeloid cell
PNAd+ HEV

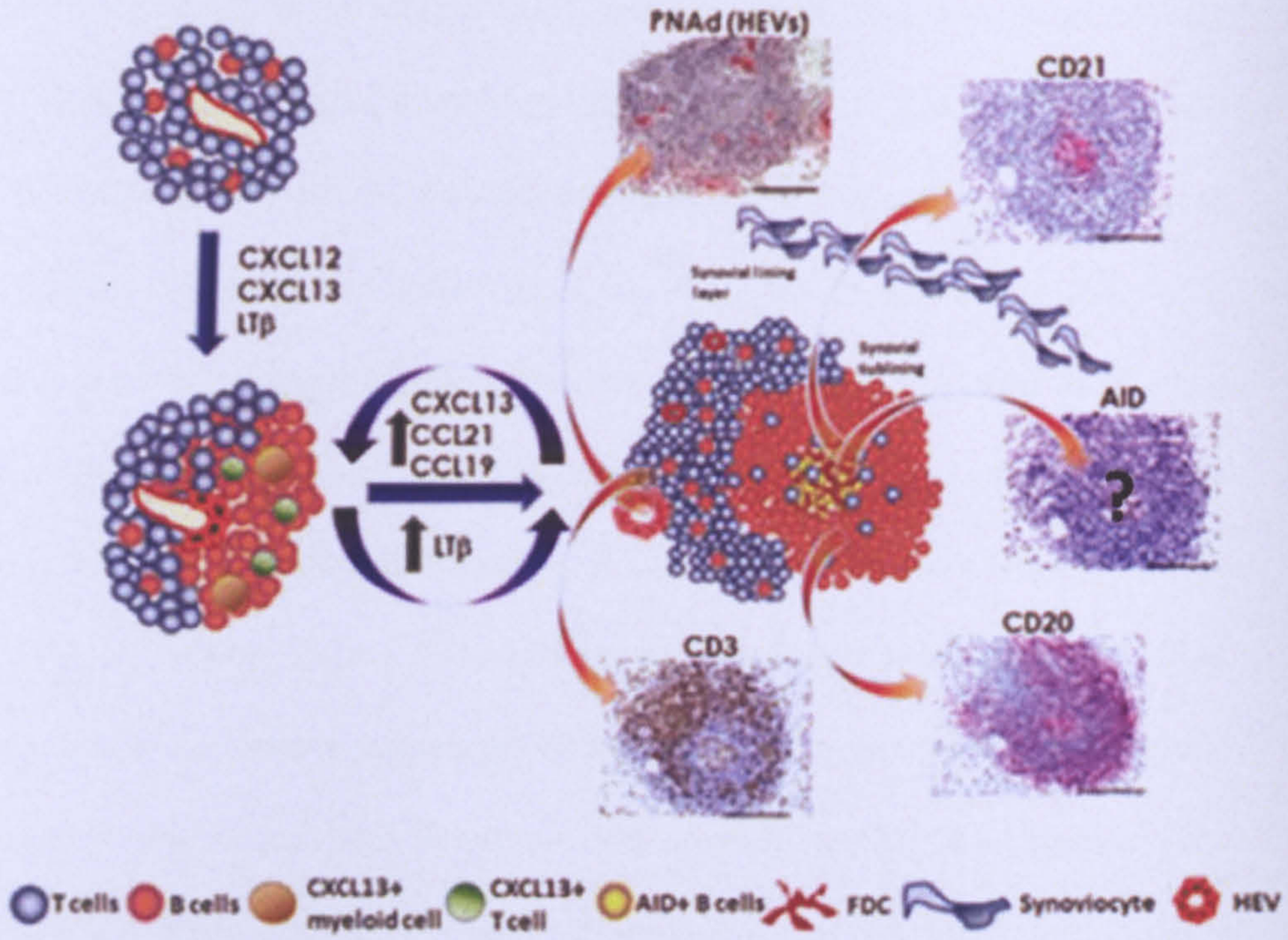

CXCL12
CXCL13
LTβ
CXCL13
CCL21
CCL19
LTβ
PNAd (HEVs)
CD21
AID
?
CD3
CD20
T cells
B cells
CXCL13+
myeloid cell
CXCL13+
T cell
AID+ B cells
FDC
Synoviocyte
HEV

Figure 11(A): Physiological lymphoid organogenesis.

The initial step of lymphoid organ development involves the interaction of hematopoietic lymphoid tissue 'inducer cells' with stromal 'organizer cells'. Triggering of LTβR leads to the production of lymphoid chemokines CXCL13, CCL21, and CCL19. Chemokines induce accumulation of further hematopoietic cells that, upon interaction with stromal cells, determine the formation of a positive feedback loop via increased signalling through LTβR and up regulation of lymphoid chemokines. This phenomenon leads to the progressive formation and enlargement of lymphomononuclear cell clusters, with the differentiation of a mature stromal architecture and high endothelial venules (HEVs). Adapted from (332).

(B): Development of tertiary lymphoid structures in RA synovium.

Initial recruitment of lymphocytes in the sublining of the RA synovial membrane displays a disorganized accumulation of T (predominantly) and B cells around a central blood vessel (G1 / G2 aggregate, top left). The increasing size of the aggregate is accompanied by variable up regulation of key molecules regulating lymphoid organogenesis and homeostasis in physiological conditions, such as LTβ and lymphoid chemokines CXCL13, CCL21, and CCL19. At this stage, CXCL13 can be produced by a subset of antigen (Ag)-experienced T lymphocytes, by newly recruited monocyte-derived cells and differentiated stromal cells. Conversely, CCL21 is expressed by smooth muscle actin stromal cells surrounding new HEVs. Expression of lymphoid chemokines can lead to initial T / B cell compartmentalization, increased accumulation of LTβ+ hematopoietic cells promoting further differentiation/ proliferation of stromal and vascular cells (G3 aggregate, bottom left). Amplification of this process in the context of an Ag-rich

environment can lead (through T-cell dependent or T-cell independent mechanisms) to the formation of functional GC-like ectopic lymphoid structures characterized by differentiation of follicular dendritic cell (FDC) networks (CD21) and possibly activation induced-cytidine deaminase (AID) positive B cells, variably associated with other lymphoid structural features (CD3, T cells; CD20, B cells) (G3 aggregate, right). Microphotographs reproduced from(417).

Pathogenic relevance

The production of autoantibodies by the synovial membrane has been demonstrated by a number of studies, in particular the observation that anti-CCP antibodies can be detected in the sera of SCID mice transplanted with RA synovium(418). Furthermore their detection within synovial fluid (419)and their demonstrated concentration at this site(420) suggests local autoantibody production. These data is further supported by clinical reports correlating lymphocytic aggregates within the synovial membrane with the presence of RF in the sera(421) and with the detection of ANAs in patients with JIA(422), suggesting a direct role for them in disease pathogenesis as the site of the production of autoantibodies. This is not the only work suggesting that ectopic GC maybe functional, an analysis of the IgH V gene repertoire of B cells microdissected from these structures demonstrated intraclonal diversity strongly suggesting an ongoing antigen driven B cell differentiation within these structures(423). However, all these observations have been questioned by two recent studies. The first(388) performed on synovial tissue obtained arthroscopically from 103 patients found not only a much lower incidence of ELN (8%) (as defined by the presence of FDCs) , but further no association between lymphocytic aggregates and the presence of autoantibodies (RF and anti-CCP) in the sera. The authors did observe higher levels of inflammatory markers and general synovial inflammation in patients with lymphocytic aggregates but did not observe increased severity of clinical symptoms and signs. The second study(416) investigated the association between ELN and the production of autoantibodies by correlating the presence of ELN within synovial tissue from 112 RA patients with the detection of autoantibodies (RF and anti-CCP) in both the sera and synovial fluid and

suggested that the lack of correlation seen did not support functionality of ELN. Interpretation of these result should consider that no conclusive data are available that determines whether ELN is stable throughout the disease process, and its heterogeneity both within and between joints. This is of crucial importance when studying small samples of material from one joint from a single time point and extrapolating this to explain pathogenic effects at different time points or distribution within the body. A point reinforced by data supporting functionality of lymphocytic aggregates by demonstrating their association with autoantibodies when examined locally within synovial tissue(424).

The observation by Klimiuk et al (425) that pathological subtype of the synovial membrane correlated with clinical phenotype suggested a direct role for these structures in disease pathogenesis. This study demonstrated a significant association with the presence of lymphocytic aggregates and higher disease activity (measured by DAS, ESR, CRP and number of swollen joints) and erosive damage in comparison to patients with a diffuse infiltrate. This clinical association may not only be explained by the local production of autoantibodies but by the differential expression of a number of key cytokines within tissue containing ELN(421).

Thus, whether the synovial membrane acts as a reservoir for long lived plasma cells or whether it is actively involved in the manufacture of autoantibodies and furthermore whether it is actively involved in mediating other pathways of RA pathogenesis is currently controversial(426).

Joint erosion

The pannus mediated degradation of cartilage and bone is thought to be primarily driven by the release of proteolytic enzymes by macrophages and fibroblasts.

MMP-1 expression within synovial tissue, fluid and within sera has been correlated to the development of erosions in both early and established RA(427;428). MMP3 has similarly been detected at elevated levels in the synovial fluid and sera of RA patients(429).

Bone resorption and hence erosion is determined by osteoclast formation and activation. The formation of osteoclasts from monocyte/macrophage precursors is primarily driven by two factors macropage colony stimulating factor and receptor activator of nuclear κβ Ligand (RANK-L), both of which are up regulated by IL-1 and TNF-α(430;431). Further, the relative levels of RANK-L along with its natural inhibitor osteoprotegerin (OPG) are known to determine osteoclast activity in RA, with increased ratios of RANK-L to OPG seen within the RA joint(432).

This crucial role for RANK-L in RA pathogenesis has been harnessed for therapeutic intervention with the use of RANK-L inhibitors, which have shown demonstrated efficacy in preventing ongoing erosive damage(433).

The synovial membrane as a prognostic marker in rheumatoid arthritis

The use of the synovial membrane as a potential prognostic tool has received attention since its recognition as the primary site of inflammation in RA. Early work on the pathological appearance demonstrated some characteristic features such as the expansion of the synovial lining layer, palisading of the synovial cells(434), the presence of multinucleated giant cells (435), the tendency of the lymphocytic infiltrate to form follicular structures(335) and haemosiderosis(436), but none of these appearances were found to be specific for RA and hence could not be used as diagnostic criteria. The subsequent development of IHC, combined with specific recognition of novel markers by monoclonal antibodies, has allowed the demonstration of a diverse cellular infiltrate within the RA synovial

membrane, with qualitative differences in histological appearances and quantitative variations in cell types between sub-groups of RA patients, differing in both prognosis(437) and response to treatment(438-442). In addition, the microscopic changes of the synovial membrane have been demonstrated in clinically uninvolved joints(443) suggesting that the more accessible knee joint could be a valid source of tissue in most patients. Technically the advent of new methodologies for the acquisition of SM, such as ultrasound guided needle biopsy, provides means that allow for the feasible acquirement of synovial tissue in a broader range of clinical settings. Added to this progress has been the arrival of digital image analysis (DIA), which allows rapid semi automated processing of tissue and has revolutionised the examination of the synovial membrane. The validation of DIA has shown it to be not only equivalent to the gold standard of manual counting(444) but more sensitive to changes within the synovial membrane than semi-quantitative analysis(445). Put together these advances illustrate the realistic potential of the synovial membrane as a prognostic marker in RA.

Current histopathological scoring systems

Early work on arthroscopically obtained synovial membrane did not suggest a promising correlation between the histological appearance of the SM and clinical features of disease(446;447), but subsequent studies on both arthroscopically and arthroplastically obtained synovial membrane have produced more positive results. An association between the presence of synovitis and degree of histological inflammation, as measured by 2 separate semi-quantitative inflammatory scores (the Rooney and the Tak score), was demonstrated in patients with early or established RA(447-449). The Rooney score(447;448) which assesses 6

histological features of the SM (synoviocyte hyperplasia, fibrosis, proliferation of blood vessels, perivascular infiltrates of lymphocytes, focal aggregates of lymphocytes and diffuse infiltrates of lymphocytes), has also been assessed in a prospective study, involving 60 patients with established RA, but this failed to show its use as a prognostic marker as no correlation was seen with joint damage progression over a 2-year period (176;447). A further semi-quantitative histological scoring system was initially developed by Koizumi et al. (447;450) to allow reliable differentiation of RA and OA synovium, it examines 8 histological features of the synovial membrane. When this score was examined in a cross sectional study in end stage RA(447;451)a significant correlation was found with the degree of erosive damage and levels of CRP, this study also found no significant correlation between the Rooney score and clinical features including erosive damage. The Koizumi score, to the best of our knowledge, has not been tested in a prospective study looking at erosive progression. In the quest for the identification of predictive biomarkers, macrophage numbers have received much attention, since an early study showed they correlated with the degree of pain (as a surrogate for arthritis activity) in the biopsied knee, in comparison to the number of CD4+ T cells(449) which did not. A further study, looking at T cell infiltration within the SM did show that an improvement in a composite clinical index of disease activity correlated with a decrease in T cell infiltration within the synovial membrane(447;452). A small prospective study of 12 patients with RA demonstrated an association between the intensity of macrophage numbers at baseline with erosive damage (453) and this finding was replicated in a later larger study involving 26 patients followed for a mean of 5.8 years(437). The number of sub lining macrophages has also been correlated with the clinical course of RA and

response to treatment (454). In early arthritis matrix metalloproteinase-1 gene expression (MMP-1) correlated with the development of erosions in a study involving 20 patients, 12 of which had RA (427). In this study macrophage number again correlated with MMP-1 expression and erosive damage over 1 year. A second larger study, however, has not confirmed the usefulness of macrophage numbers in predicting outcome in early arthritis(455), suggesting numbers of granzyme B+ cytotoxic cells, T cells, and FLS as better correlates of erosive damage, the authors suggested patient selection and trial numbers as reasons for these conflicting results.

Current therapies for rheumatoid arthritis

In recent years the goal of treatment in RA has been to achieve disease remission, which is an achievable goal currently in around 20-40% of patients with early arthritis, but a more difficult aim in those with established disease. The most commonly used DMARDs are methotrexate, sulphasalasine and hydroxychloroquine and evidence now overwhelmingly supports the use of these drugs in combination in early RA to prevent the progressive erosive damage that equates with disability(456-459). Alternative DMARDs include leflunomide, a specific pyrimidine inhibitor developed for use in RA. In addition therapies such as gold, cyclosporine and azathioprine are being used with much less frequency, due to a comparative lack of efficacy and poor side effect profile.

The advent of biologic therapies, however, has revolutionized the treatment of RA. Currently the most commonly used biologic therapies inhibit TNF: infliximab consists of a chimeric antibody to TNFα, adalumimab a humanised antibody to TNFα and etanercept consists of a TNF- receptor fusion protein that binds LT in addition to TNF. Data from a number of studies now support the use of anti-TNF

therapy in combination with methotrexate for maximal therapeutic efficacy and importantly the advent of anti-TNF therapy has demonstrated the realistic possibility of clinical remission (460-464).

A more recently developed biologic therapy, abatacept, has a licence for use in RA. It inhibits T cell activation via the inhibition of the CD28-CD80/CD86 co-stimulatory pathway and consists of a recombinant fusion protein comprising CTLA4 linked to a modified Fc portion of human IgG_1. Trials have demonstrated it to be efficacious in combination with methotrexate in patients known to have failed anti-TNF therapy, the ATTAIN study(465), as well as biologic naive patients and further to have superior efficacy when compared to infliximab, the ATTEST study(466).

The use of B cell depletion therapy in RA is discussed on page 112 along with the more recent development of agents aimed to modulate B cell survival and activation. In addition there are a number of drugs either in development or newly licensed that aim to interrupt critical cytokine pathways involved in RA pathogenesis, and these are discussed within Cytokines (page 64).

B cells in rheumatoid arthritis

The presence of autoantibodies such as RF and anti-CCP in the sera of RA patients has long suggested a role for B cells in the pathogenesis of RA, and a central role in disease pathogenesis has now been confirmed by the demonstrated efficacy of the B cell depleting agent rituximab (467). It is now recognised that the role of B cells is not just limited to autoantibody production, but that they are efficient antigen presenting cells, mediators of T cell activation and producers of pro inflammatory cytokines.

Autoantibodies in rheumatoid arthritis

Rheumatoid Factors (RFs)

RF was first described in 1940 by Waaler (11) and as such RA was the first characterised autoimmune disease. RFs are antibodies directed against the Fc portion of IgG and are relatively sensitive markers of RA, but lack specificity being detected in up to 10% of healthy individuals as well as in other inflammatory diseases such as Sjogren's syndrome and SLE as well as in infections such as TB. RFs produced outside the context of RA however are generally of low affinity and lack the characteristic high ratio of replacement to silent mutations seen within CDRs of antibodies having undergone affinity maturation. It is thought that these RFs are produced primarily by CD5+ B cells. In comparison, work isolating RF producing B cells from rheumatoid synovium has demonstrated a high ration of replacement to silent mutations thus suggesting that these antibodies have undergone a T cell dependent affinity maturation process(468;469).

RF has been demonstrated to be capable of enhancing tissue destruction in RA, by the formation of immune complexes and subsequent complement fixation. Furthermore the ability of RF producing B cells to recognise, process and present antigen that would otherwise not be recognised by T cells theoretically could lead not only to the recognition of self as auto antigens but the perturbation of the inflammatory cascade by the development of the autoreactive B cell (Figure 12). Moreover, high titres of RF associate with a higher degree of erosive damage (150;470)and thus there is a clinical indication that they may be directly involved in disease pathogenesis.

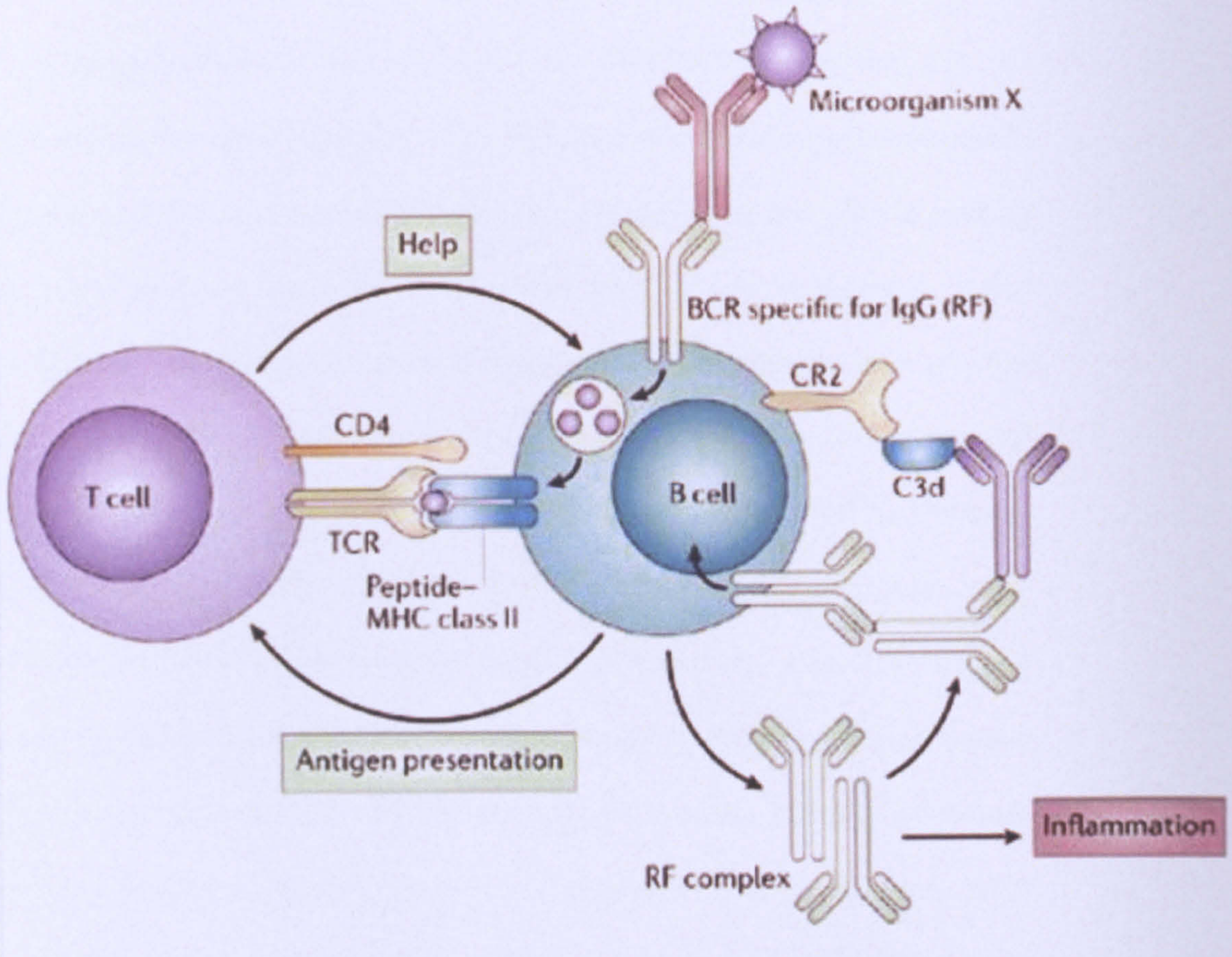


Figure 12: Self perpetuating B cells

IgG rheumatoid factor (RF)-secreting B cells provide the simplest example of how autoreactive B cells might become 'self-perpetuating'. A B-cell receptor (BCR) specific for IgG (RF) can mediate the endocytosis of foreign antigens and acquire help from T cells specific for such antigens following antigen presentation. Soluble IgG RF can polymerize and acquire complement component 3d (C3d), which can amplify re-afferent (feedback) signals through BCR of RF specificity by co-ligation of complement receptor 2 (CR2). IgG–RF complexes are also pro-inflammatory. TCR, T-cell receptor. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology ((471), copyright 2006)

Anti-citrullinated protein/peptide antibodies (ACPA)

In 1964 Niehuis et al were the first to the report the detection of a new class of autoantibodies within the sera of RA patients, distinct from RF, in that they were able to bind to perinuclear granules in normal buccal mucosa(472). They named this antibody the perinuclear factor (APF). This was followed by a subsequent report in 1979 documenting the presence of autoantibodies reacting to the keratinized epithelial layer of rat oesophagus in the sera of RA patients, anti-keratin antibodies (AKA)(473). It was some 15 years later before it was recognized by 2 separate groups that both APF and AKA recognized the same epitope(474;475), and further that the conversion of arginine to citrulline was required for such specificity(476;477). This post translational modification is now known to be dependent on the enzyme peptidylarginine deiminase (PADI)(476;477). More recently an additional antigen specific for RA has been identified, known as Sa antigen(478), and this has now been shown to be dependent on citrullination of the cytoskeleton protein vimentin(479). It is now known that citrullinated proteins can be found in a number of inflamed human tissues(480) as well as in joints of animal models of arthritis(481;482), although neither of which in general lead to the production of specific antibodies . It therefore appears that the subsequent development of anti-citrullinated peptide/protein (ACPA) antibodies, rather than dysregulated citrullination, is highly specific for RA.

Antibody reactivity has now been shown to be directed to a large number of other citrullinated proteins and peptides such as deiminated forms of α and β fibrin(482;483), citrullinated fibrinogen (484)and citrullinated peptides of collagens I and II(482;485).

Initial assays for the detection of ACPA used linear stretches of citrullinated proteins, but these proved difficult to standardise and a cyclic citrullinated peptide (CCP) test was developed, known as the anti-CCP1 test(486). This was followed by a second generation assay, anti-CCP2, generated from a large library of citrullinated peptides/proteins, and known to have improved sensitivity (64-89%) and specificity (88-99%) compared to the first generation test(487).

Anti-CCP antibodies have been detected in the sera up to 14 years prior to diagnosis of RA, raising the concept of an early break in central tolerance. They are a particularly useful tool in early arthritis to predict those patients, who though not yet fulfilling the current ACR diagnostic criteria, will go on to develop RA and in combination with RF have been shown capable of predicting RA with a sensitivity of between 30-39% and specificity of 98-100% . This is of crucial clinical relevance as it is now clear that there is an early window of opportunity to induce disease remission(133;488) and thus the early identification of patients with poor prognostic features is of critical importance.

Anti-CCP antibodies have further been demonstrated to be superior to RF in the identification of patients with greater disease activity, as measured by swollen joint counts, physicians global assessment of disease activity ESR and CRP (489)and to correlate with a higher erosive burden(152;490). They have also been shown to fall in line with effective therapy(491). These data along with their pre clinical detection suggests a direct role for them in disease pathogenesis. Their link with the SE and smoking is discussed on page 26.

Type II collagen (CII)

CII was first proposed as a putative autoantigen more than 30 years ago, and its identification within articular cartilage as well as its ability to induce an erosive

arthritis in experimental animals provided further support for this hypothesis. Subsequently antibodies to both native and denatured collagen have been identified in the sera of 10- 30% of RA patients(492;493), both in the synovial fluid and within synovial tissue(494). They also appear early in the course of the disease and have been suggested to be associated with a poor prognosis(495). Up to 37% of healthy individuals also have T cell responses to CII(496), and there have further been no consistent reports of associations with clinical markers. The use of oral collagen as a therapeutic tool to induce tolerance has also met with limited success(497). Thus the role of anti-CII responses in RA pathogenesis at present is unclear.

Nuclear ribonucleoprotein complex (anti-RA33/anti-hnRNP A2)

RA33 is a component of the spliceosome and antibodies to this complex were first reported in 35% of RA patients nearly 20 years ago(498). They have subsequently been shown to be present in other autoimmune mediated diseases, such as SLE and mixed connective tissue disease(499). Anti-RA33 has also been identified in TNF-transgenic mice (500)and MRL/lpr(501) mice, both used as animal models of arthritis. Differences in antibodies detected in patients with RA/SLE in comparison to MCTD patients have been demonstrated to be a result of conformation differences in the antibody binding sites(502) which is interesting as their detection in SLE appears to correlate with an erosive phenotype(503). No predictive value for these antibodies with regard to disease severity or progression in RA has been demonstrated(504) despite their detection in the sera early in the course of the disease and thus their measurement is not part of routine clinical practice.

Antibodies to calpastatin

Calpastatin is the endogenous inhibitor of calpains which are intracellular calcium-activated cysteine proteases. Calpains have been demonstrated to be over expressed in the synovial tissue of RA patients(505;506) and capable of degrading components of articular cartilage(507). Antibodies have been described in the sera of patients with a number of systemic inflammatory diseases albeit at a significantly higher titre in RA patients(508;509). It is possible that antibodies to calpastatin interfere with its ability to inhibit the action of calpains(510) and some *in vitro* work supports this hypothesis in that calpains are released from the calpain-calpastatin complexes in the presence of RA sera positive for anti-calpastatin antibodies(509).

Heat shock proteins (HSP) including BiP

HSP or stress proteins are intracellular molecules with a number of housekeeping and cytoprotective functions. These molecules are both constitutively expressed and capable of being induced by a number of triggers, including oxidative stress. They are also now known to be capable of both pro and anti-inflammatory modulation(511). Many groups have reported the detection of antibodies to HSPs in RA, although their detection does not appear to be disease specific(512). More recently the HSP BiP has been identified in both RA patients and experimental models of arthritis(513-515). BiP is a 78kDa glucose regulated endoplasmic reticulum chaperone that has been identified within the RA joint and in the sera of RA patients(516). Further BiP has demonstrated *in vitro*(517) and *in vivo*(518) immunomodulatory capacity and is therefore currently under investigation as a therapy for RA(519).

Anti-glucose 6 phosphate isomerase (G6PI) antibodies

The search for anti G6PI antibodies in RA patients was generated from their identification in the K/BxN mouse model of arthritis, which in many clinical and pathological respects mirrors RA(520). Of particular interest in this animal model was that the transfer of even small amounts of serum from K/BxN mice induced arthritis, even in non susceptible mice(521). G6PI is a ubiquitously expressed glycolytic enzyme and is not joint specific. Reports of detection in RA patients have been variable, with an initial report suggesting a sensitivity of around 64% and a high specificity(522). This was followed by work from a number of other groups that was unable to replicate the original data, explained by a contamination of the commercial G6PI preparation used in the initial study by other proteins which reacted with antibodies in RA sera(523-526). More recent work has suggested that anti-G6PI antibodies may be specific for RA patients with Felty's syndrome being identified in the serum of 12/13 patients tested(527). It remains to be determined whether such sero positivity reflects general dysregulation of autoimmunity in Felty's syndrome or whether anti G6PI antibodies are specific and indeed whether such results can be replicated in other patient cohorts.

Autoantibodies as mediators of RA pathogenesis

Several antibodies have been associated with RA in humans, including RF, G6PI and more recently ACPA. There is now evidence supporting a role for autoantibodies not just as markers of disease but as mediators of disease pathogenesis. Clinically both ACPA and RF are associated with a worse prognosis(470;528-530) and both are present years before disease diagnosis(531;532). In addition ACPA are highly specific for RA(529) and both PADI (533)and citrullinated proteins (534)have been identified within the synovial

membrane. Furthermore, animal models have demonstrated that autoantibodies are capable of both initiating (although not maintaining) disease(521;535;536). RF is thought to enhance tissue damage via formation of immune complexes, activation of complement and thus recruitment of inflammatory cells to the synovium. ACPA are also now thought to be directly pathogenic as work in the CIA mouse model has demonstrated their capacity to enhance collagen induced tissue destruction(481). In addition to the formation of immune complexes and activation of complement, antibodies are also capable of mediating tissue damage via Fcγ receptor (FCγR) activation. This pathway has been demonstrated to be critical to disease development in the CIA model of arthritis as mice deficient in the inhibitory FcγRII exhibited more aggressive disease(537). A further effector function of autoantibodies appears to be the C5 component of the complement system. Data have demonstrated that both the CIA and K/BxN models of arthritis are dependent on the C5 for disease expression and further that administration of C5 antibodies in the K/BxN model ameliorates disease(538;539).

Autoantibody independent roles for B cells in RA pathogenesis

B cells are one of a number of antigen presenting cells that are capable of processing antigen and presenting it to T cells via MHC class II molecules and are thought to be the predominant APC in later phases of the immune response(540). This ability to process and present antigen permits RF producing B cells to process antigen contained within the RF-Ag complex that would not otherwise be presented to T cells. In addition IgG RF has the unique ability to cross link and form immune complexes with C3d, and this cross linking has the potential to override negative signals from soluble self proteins. Thus, RF

producing autoreactive B cells have the potential to become self perpetuating (541) (Figure 12).

Animal models have also provided evidence to support a role for autoantibody independent B cell activation in RA pathogenesis. Evidence in the proteoglycan induced model of arthritis has demonstrated a specific requirement for B cell induced T cell activation in disease induction, a role that could not be compensated for by other antigen presenting cells(542). Work in the Hu-RA SCID mouse model has also demonstrated a critical requirement for B cell induced T cell activation in the induction of ectopic lymphoid structures(543).

Finally, recently reported data have demonstrated a role for TLRs in B cell activation, in particular TLR9. This pathway was shown to be activated by the simultaneous activation of TLR9 and BCR by chromatin containing immune complexes and resulted in the production of RF(161) thus demonstrating a critical link between the innate and adaptive immune systems in the development of RA. This work is supported by data on TLRs from animal models of arthritis, with TLR4 deficient mice exhibiting milder disease in the KBxN model of arthritis(544) and the induction of arthritis by intra articular injection of CpG DNA or double stranded RNA(545).

A key development in B cell biology has also been the recognition that B cell deficient mice develop an exacerbated experimental encephalomyelitis, when compared to wild type animals, suggesting a protective role for B cells in autoimmune mediated disease(546). The protective subtype appears to be a result of IL10 producing B cells, named regulatory B cells, that have been shown capable of ameliorating CIA via adoptive transfer experiments (547). How regulatory B cells regulate the immune response is currently unknown, they may act either

through a restoration of Th1/Th2 balance, or a direct modulation of effector cells, such as macrophages or DCs. Finally, IL10 producing cells have also been suggested to function as secondary antigen presenting cells which could result in an abortive response and induction of anergic CD4+ T cells, or perhaps act via the recruitment and/or induction of differentiation of regulatory T cells (reviewed in(548)).

Pharmacological modification of B cells in rheumatoid arthritis

B cell depletion therapy

The efficacy of the B cell depleting agent rituximab in RA was first reported by Edwards et al in 2001(549). The rationale behind its use was based on the concept that the removal of memory B cells may lead to a resetting of the immune system and hence induction of disease remission. Rituximab is a chimeric monoclonal antibody against the CD20 molecule that was first licensed for use for Hodgkin's lymphoma in 1997. CD20 is expressed on all stages of B cell development, except at the very early pre B cell stage and on terminally differentiated plasma cells (Figure 13) and is a particularly useful target as it is not shed or internalized on binding its antibody. Killing of B cells is thought to be mediated by 3 mechanisms, antibody dependent cytotoxicity, apoptosis and complement mediated lysis. Animal studies have shown that killing mechanisms differ in different microenvironments, and further that specific sites such as the splenic MZ and GC B cells within Peyer's patches are particularly resistant to killing(550). Although treatment with rituximab is invariably associated with near complete peripheral B cell depletion, animal studies have demonstrated that this is not the case for solid tissues such as lymph node(551). Further variable degrees of depletion have been shown within the synovial membrane in patients with RA in a number of studies

perhaps explaining the differing clinical responses observed(390;552;553). In addition recent data have challenged the concept that complete peripheral depletion is achieved in all patients, and has demonstrated - using highly sensitive FACS analysis - that detectable circulating B cells following initial rituximab infusion is followed by a poor clinical response (554).

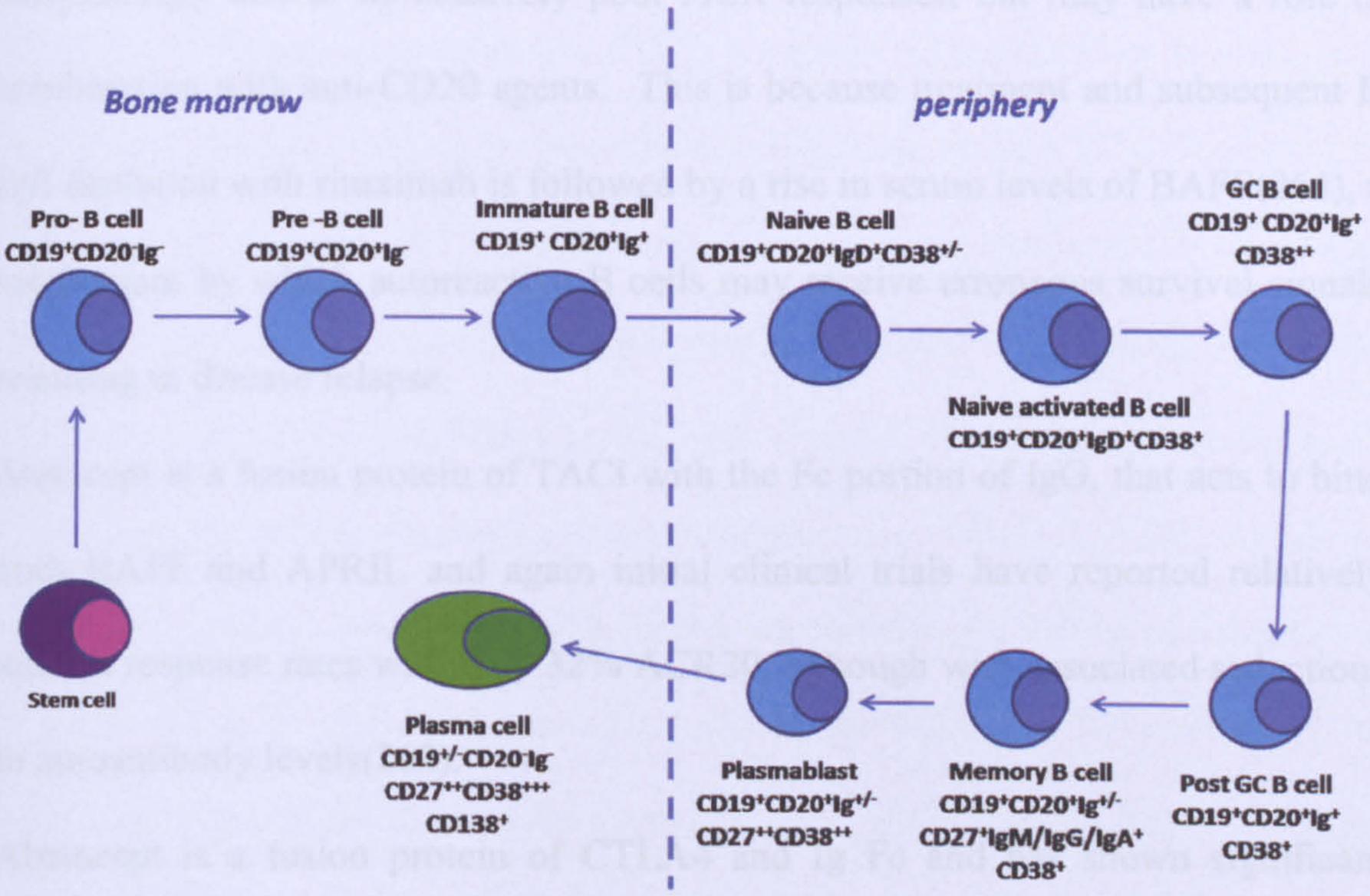
Other possible mechanisms for resistance following rituximab therapy in RA comprise drug pharmacokinetics, including penetration of structures such as lymph nodes and synovium, FCγR polymorphisms(555), local and systemic expression of B cell survival factors and resistance of specific B cell subsets (in particular marginal zone and follicular B cells)(550).

Rituximab has been shown to be efficacious and safe when used in combination with methotrexate(467;556;557) and is currently licensed for use in clinical practice for patients who have failed one anti-TNF therapy. Rituximab is not administered continuously but rather given as 2 doses at an interval of 2 weeks, with repeat courses given at disease relapse. At present the current mean time to B cell repopulation is 6 months. Repeat treatment appears to be as efficacious as primary therapy, and safety data shows no excess morbidity with up to 4 treatment courses(558).

In addition fully humanised antibodies to CD20 are currently in clinical development and include Ocrelizumab, a molecule with demonstrated efficacy in phase II clinical trials(559).

Figure 13: B cell development pathways.

Stages of B-cell maturation are indicated by their anatomical site and the expression of cell-surface markers, including the target for rituximab, CD20. GC= germinal centre. Adapted from(471)



Other therapies targeting B cell survival or activation

In addition to directly depleting CD20+ B cells a number of other biological therapies are currently either in clinical use or in development that target B cell survival or activation (14) and are summarised in Figure 14.

Belimumab is a fully humanised monoclonal antibody specifically targeting BAFF and has been shown to have partial efficacy in treating RA which was associated with a fall in autoantibody levels(560). Belimumab is unlikely to be used as monotherapy due to its relatively poor ACR responses, but may have a role in combination with anti-CD20 agents. This is because treatment and subsequent B cell depletion with rituximab is followed by a rise in serum levels of BAFF(264), a mechanism by which autoreactive B cells may receive erroneous survival signals resulting in disease relapse.

Atacicept is a fusion protein of TACI with the Fc portion of IgG, that acts to bind both BAFF and APRIL and again initial clinical trials have reported relatively modest response rates with only 32% ACR20, although with associated reductions in autoantibody levels(262).

Abatacept is a fusion protein of CTLA4 and Ig Fc and has shown significant efficacy in RA (465). Abatacept selectively modulates the CD86 or CD80-28 co-stimulatory signal required for full T-cell activation. CD80 or CD86 on the antigen presenting cell binds to CD28 on the surface of the T cell which facilitates activation. CTLA4 is a naturally occurring inhibitor that is induced on the surface of the T cell, but has a higher affinity for CD80/86 than CD28, therefore abatacept modulates T cell activation by exploiting this pathway. In addition the expression of CD80/86 by activated B cells further modulates T dependent B cell activation(561), supported clinically by a reduction in production of

autoantibodies(465) in clinical trials of abatacept. It would appear also that memory B cell production is predominantly inhibited by CTLA4-Ig(561).

Epratuzumab is an unconjugated humanised monoclonal antibody that recognizes a pan B cell marker (CD22) with a similar distribution to CD20, being found on all B cells past the pre-B cell stage to the pre plasma developmental stage. The function of this monoclonal antibody to CD22 is unlike that of CD20, in that it can deliver intracellular signals, either constitutively, or via interaction with its ligand which is an α2,6-sialic acid residue found in many glycoproteins, including IgM and other cell surface proteins. As CD22 is rapidly internalized by B cells, it is thought to be a relatively poor target for B cell killing, unlike CD20.The effect of CD22 signaling is generally, but not entirely negative or anti-stimulatory. It also modifies signaling through other cell surface molecules including the BCR, CD19/21 and CD45. CD22 appears to have a direct link to the development of autoimmunity, which has been predominantly explored in SLE. CD22 knockout mice have a hyper responsiveness to BCR cross linking, and a reduced response to T independent antigens. There is also evidence linking CD22 polymorphisms in mice and humans to the development of SLE. Epratuzumab might therefore modify the action of B cells without killing them, explaining some of the clinical effects seen that are not accompanied by complete B cell depletion. It does not block interaction with its ligand, but in fact initiates signaling through the molecule. Therefore in early clinical trials of the drug in SLE, only partial depletion of B cells were seen and no effects on complement or autoantibody levels(562). Additionally early trials in Sjogren's syndrome have reported a clinical benefit, with 53% of patients achieving a clinical response(563), partial B cell depletion and no effect on serum Ig levels. So far no reports have been made

of the drug in RA, although a role in addition to rituximab has been postulated as this has shown some efficacy in treatment of lymphoma(564).

Anti-CD40L blockade (IDEC-131- and BG9588), and hence blockade of an arm of the adaptive immune system, has been explored in SLE however trials have been halted due to a pro thrombotic effect of the drug. However positive effects were seen on levels of autoantibodies, including ds-DNA(565). A further drug that has been developed for use in SLE, raises an alternative approach attempting to selectively deplete pathogenic B cells. This drug (LJP 394) attempts to cross link BCRs on DNA-specific B cells in SLE patients in such a way as to result in a negative tolerising signal, however initial clinical trials have not produced promising results(566).

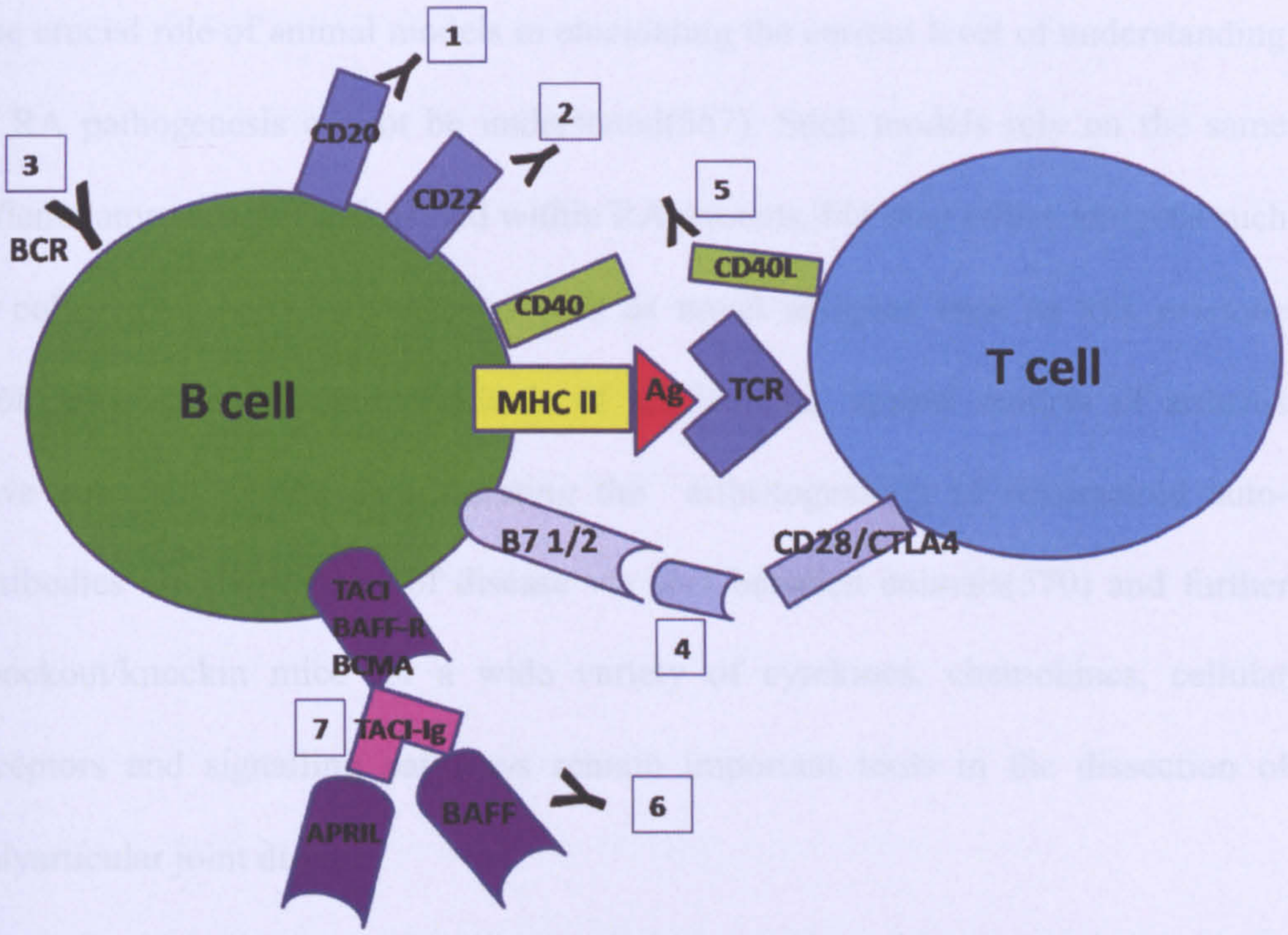


Figure 14: Current and potential therapeutic targets modulating B cell responses in RA

(1) B-lymphocyte depletion with rituximab anti-CD20, (2) epratuzumab anti-CD22, (3) LJP 394/abetimus; (4) blockade of T-cell co stimulation CTLA4-Ig (abatacept) (5) blockade of B-cell stimulation via CD40L with IDEC131 and BG9588, (6) belimumab an anti-BAFF monoclonal antibody (7) blockade of B cell survival and activation with TACI-Ig (atacicpet). Adapted from (4)

Preclinical animal models of Rheumatoid arthritis

The crucial role of animal models in elucidating the current level of understanding of RA pathogenesis cannot be understated(567). Such models rely on the same inflammatory cascades identified within RA patients, but may utilise antigens such as collagen or peptidoglycans, as well as novel antigens such as xxx *pristane* (568;569). Amongst their multitude of applications, animal models of arthritis have been key in the demonstrating the arthritogenicity of rheumatoid auto-antibodies via the transfer of disease via sera between animals(570) and further knockout/knockin mice for a wide variety of cytokines, chemokines, cellular receptors and signalling pathways remain important tools in the dissection of polyarticular joint disease.

The induction of an auto-immune response to antigen is a common trigger in animal models of arthritis, however it should be noted that the type of immune response is important(571). The induction of inflammation into the joint need not necessarily lead to a sustainable destructive arthritis. Each type of inflammation occurs as a consequence of the activation of a different arm of the immune response, namely type of hypersensitivity as classified by Gel and Coombs. Each type may be induced in mice using a combination of three basic ingredients: Animal strain; Antigen; and adjuvant. Adjuvants are agents added to antigens that enhance the translocation of antigen to the lymph node, enhance retention at the site of injection to prolong delivery of the antigen, induce local reactions involving mast cells and Th cells to enhance chemokine synthesis, induce local cytokine synthesis and systemic cytokines to increase the circulating population of immune cells, and stimulate the innate response through accessory cell pattern recognition receptors (Toll receptors). The latter serves to direct the immune response towards

Th1 cell mediated immunity and IgG synthesis, diverting it from Th1 IgE dominated allergic responses.

There is a sensitisation step to an antigen, during which antigen presenting cells process the antigen and present it (or them) to T cells to produce cell mediated immunity. T cells also co-operate with B-cells to produce an IgG humoral response. The fixation of complement by these antibodies, and their binding to Fc receptors (FcγR) that can regulate neutrophil as well as macrophage function can be a key event in disease initiation and joint inflammation occurs thereafter. Since individual animals may succumb to disease at different times, the initiation of the disease may be synchronised by a boost injection comprising either the antigen in adjuvant, or a toll receptor agonist such as lipopolysaccharide (LPS).

Sampling of tissues for gene or cytokine analysis is one the prime advantages for the use of animal models. In models of arthritis, sampling serum, synovial tissues, spleens, lymph nodes, synovial washouts or even whole joints provides very useful pathobiological information.

A number of animal models are described below, but the list is by no means exhaustive. Importantly, for the purposes of this thesis, the SCID-Hu RA mouse model is described in detail.

Rat Adjuvant Arthritis

Adjuvant arthritis takes its name from the agent that induces it, Freund's complete adjuvant (FCA). This is 85% mineral oil (Bayol F) and 15% emulsifier (Arlacel-A), complete with finely ground heat-killed mycobacterial fragments. This is commonly *Mycobacterium tuberculosis* (*M.tb.*) strain H37ra, or *Mycobacterium butyricum* (*M.bt.*). This is administered at the base of the tail. Tracking of the mineral oil and mycobacterial particles shows that they distribute quickly

throughout the body, and accumulate in lymph tissues. A powerful systemic invasive inflammatory periarthritis is induced that involves not only the joints, but the formation of granulomas within meninges, skin, bone marrow and eyes, as well as in the connective tissue. The animals lose body mass and the predominant feature is a profound and crippling inflammation of the hind paws. The joints of the ankle and paws become seriously eroded, but the knees are less involved. The disease is accompanied by splenomegaly, increased gut permeability and liver involvement(572).

Adjuvant arthritis is an auto-immune disease, since it can be passed to non-arthritic irradiated recipients through T cell transfer from diseased animals(573;574), these T-cells express CD4 and the α,β-TCR(575). Susceptibility is conferred by genes within the MHC class II region(576). It is not transferred by serum, but serum from adjuvant arthritis rats can induce a flare in rats with cyclophosphamide attenuated disease, whilst serum from the cyclophosphamide animals does not(577). The MHC phenotype is weaker than that for the rat collagen arthritis, but exerts a strong influence on the severity of disease(578;579). Thus non-MHC phenotypes have a significant contribution to play. The MHC gene is *Aia1* found on chromosome 20. Non-MHC quantitative trait loci include *Aia2* and *Aia3* on chromosome 4(579). Conserved synteny among rats, mice and humans, suggests that *Aia1, Aia2* and *Aia3* contain candidate genes for several autoimmune diseases including diabetes (*Aia3* : IDDM9), SLE, inflammatory bowel disease, asthma/atopy, multiple sclerosis, and RA (*Aia3* : RA2)(579).

Histologically, the predominant cell is the neutrophil, the synovial lining is destroyed. At initiation, fibrin is deposited along the bone shafts followed by fibrin deposition in the joints. The striking feature is a strong periosteal inflammation

comprising monocytic cells that progresses to invade the joints. Bone erosion is both osteoclastic and due to the inflammatory tissue(572). Osteoblastic production of new perisoteal bone can be seen with osteoclastic erosion of old bone. Articular cartilage remains relatively untouched, though dead cartilage can be seen as associated with neutrophils or macrophages. Bone is ultimately eroded away from under the cartilage leaving it supported by fibrocartilage. At late stages fibrosis occurs with ankylosis. Lymphocyte aggregates and plasma cell infiltrates are not seen. Whilst a polyarthritis, there is special focus on destruction of the ankle joint(572).

Numerous candidates for the endogenous antigen involved in adjuvant arthritis have been proposed(580;581). Muramyl dipeptide induces adjuvant disease similar to adjuvant arthritis (582;583) and heat shock protein-65 (HSP-65) is also considered to be a major endogenous antigen(580;584). Susceptible rats do not appear to be fully tolerant to rat HSP65, and HSP65 pre-treatment induces disease tolerance(585-587).

Rat Collagen Arthritis.

This model, discovered accidentally by Trentham and colleagues (588) during attempts to generate anti-type II collagen (CII), was the first collagen derived arthritis which was followed by the mouse version some years later. Whilst similar to adjuvant arthritis described above, it is milder and often preferred for this reason(588). The arthritis is induced by the sensitisation of susceptible rat strains to native heterologous collagen emulsified in a mineral oil, namely Freund's incomplete adjuvant (FIA)(589). The inoculum can be injected i.d. in several places on the lower back, or at the base of the tail. Old protocols utilised sensitisation in the footpad, but this is now discontinued on concerns for animal

husbandry. Some protocols utilise a boost, but arthritis is elicited between 11 to 14 days reaching a maximum at around 20 days. A boost at 20 days can be given to establish a long term erosive disease(590-592).

Like adjuvant arthritis, it appears that fibrin deposition and a periostitis occurs prior to inflammation within the joint space. Fibrin is deposited over the fat pad, soft tissues and cartilage, followed by synovial hyperplasia, and infiltration by neutrophils and monocytes. The periostitis and intense mononuclear accumulation is associated with areas of ligament attachment, which then moves into the synovial space leading to invasion of the joint space, with an influx of CD90+ CD4+ Ly2- (CD8-) Ia (MHC class II) expressing T –cells, with little evidence of suppressor T cells, and subsequent destruction of cartilage and bone(588). The pattern of erosions differs to adjuvant arthritis, being less severe, but involving both the ankle and midfoot(588).

A hallmark of the CIA rat model of arthritis is the dominant B cell component of the immune response, which is specific for triple helical epitopes(591;593). The B cells generated are autoreactive and produce arthritogenic autoantibodies(594;595). There is also evidence for a critical role for T cells in disease pathogenesis, however(596;597) the relative importance of T and B cells during the effector phase of the disease is at present unclear(572).

An interesting observation in rat CIA is the presence of citrullinated proteins in the synovium, along with the enzyme responsible for citrullination, peptidyl arginine deiminase-4. This study further demonstrated the capacity of citrullination to break B cell tolerance to the endogenous antigen, rat serum albumin and correlated the levels of both PAD4 and citrullinated proteins with severity of inflammation(598).

Mouse Collagen induced Arthritis.

Mouse CIA was developed from the original rat model of CIA, due to the use of this species in gene technology(599). As in the rat, a polyarthritic response is seen, but different sources and batches of collagen coupled with different strains of mice, results in different patterns of disease. It is initiated in susceptible strains, namely the DBA1/J and NFR/N mice (haplotype H-2q) and the C57.B10RIII (H-2r), with better responses seen in males. Heterologous collagen is used, usually either bovine or chick type –II collagen to initiate disease. In addition, as in the rat, the type and form of the collagen is important. CI does not induce the arthritis and neither does denatured CII(588;599). Contrary to the situation in rat CIA, in which either incomplete or complete Freund's adjuvant can be used to trigger disease(589), in mice disease induction usually requires the presence of heat killed mycobacterium tuberculosis(599). 3-4 weeks following disease initiation with CII and CFA a severe polyarthritis enthuses with a subsequent remission of the disease after 2-3 weeks. In addition the use of autologous, rather than heterologous CII results in a disease characterised by a more chronic nature(592;600). The generation of mouse CIA is known to require both T and B cells, with the adoptive transfer of T helper cells alone unable to reliably confer disease(601;602) and overwhelming evidence indicating a critical role for anti-CII antibodies in disease initiation(603-606).

Murine CIA, comparable to human RA, appears predominantly a Th1 driven disease. Evidence for this concept comes from studies demonstrating that manipulation of the Th1/2 balance, via the administration of IL4 or IL10 (607-609) or the blockade of IFN-γ(610;611) which results in a therapeutic effect.

A common feature of RA is the presence of RF and anti-citrullinated peptide antibodies (ACPAs). CIA mice do not express rheumatoid factor. However CIA DBA/1 mice have been demonstrated to generate antibodies to citrullinated epitopes and furthermore this model has demonstrated the capacity of ACPA to enhance disease(481). Citrullination of CII has been shown to enhance its antigenicity and interestingly transgenic mice with the human HLADRB1 shared epitope, have an exaggerated CD4 T-cell response when exposed to peptides that are citrullinated at the shared epitope specific recognition sites(111). Mouse CIA may be unique amongst the mouse models in exhibiting ACPAs.

Passive collagen antibody induced arthritis

Passive transfer of collagen arthritis (termed collagen antibody induced arthritis [CAIA]) can be performed with anti-type II collagen monoclonal antibodies, a mixture of which includes the complement binding IgG2a(606;612). Commercially available kits recommend the use of DBA mice along with LPS for enhanced expression of disease. CAIA can be induced in T and B cell deficient mice, though the effect is less severe(612). The administration of CTLA4-Ig, capable of interrupting T-cell APC interactions, does attenuate CAIA if administered in the chronic phase of disease(613). Thus there is evidence that auto-antibodies in turn induce a secondary T cell reaction that further exacerbates the disease. Neutrophils are also important mediators of disease pathogenesis as their depletion reduces the severity(612) and a massive neutrophilic infiltration of synovium is found within joints of diseased animals(612). IgG FcγR engagement is also very important. Knockout mice for the FcγR chain are resistant to disease. FcγRIII knockout mice have reduced severity(614), whilst absence of the inhibitory form, FcγRIIb, exacerbates CAIA disease(615). Complement is also a

factor, C3 depletion with cobra venom factor reduces the response(616), as does C5 knockout(617).

K/BxN Arthritis

The group of Mathis and Benoist fortuitously discovered that T-cell receptor transgenic mice - recognizing an epitope of bovine RNase (KRN) - crossed with non-obese diabetic (NOD) mice, generated offspring (K/BxN mice) that develop a spontaneous arthritis resembling human rheumatoid arthritis(520). The most recent of the major arthritis models discovered that the knowledge from previous models described above, and the application of transgenic, biologic and analytical technologies has meant that much has been learned within a short space of time. The onset of arthritis is observed at 3-4 weeks of age presenting clinically as a chronic, progressive, polyarthritic disease with a distal to proximal gradient of severity(520). The transgenic TCR recognizes glucose-6-phosphate isomerase (GPI) as the antigen presented by NOD antigen presenting cells in the context of MHC class II molecule I-A^{g7}. It was this finding in the K/BxN model that led to several studies investigating the role of GPI antibodies in humans, although the data generated from these studies currently remains somewhat controversial(524;527). This molecule is also recognised by antibodies generated in the murine CIA. It was this ubiquitously expressed glycolytic enzyme that is identified to be the crucial driver for T-lymphocyte initiation and B-lymphocyte production of arthritogenic immunoglobulins that leads to disease in the offspring. Early T-lymphocyte requirement is determined by the fact that administration of anti-CD4 neutralising antibodies prevent disease but only if given at least 5 days before the disease onset(618). Furthermore, KRNxNOD mice lacking B-lymphocytes do not develop arthritis, confirming the necessity of this cell type for

initiation of disease(619). The effector phase of the disease has been demonstrated to be initiated by the binding of immunoglobulins to joint surfaces(620).

Antigen –induced arthritis.

Originally described in the rabbit using bovine serum albumin, this model is now used in the rat and more commonly in the mouse. Animals develop an inflammatory arthritis when primed with an antigen (e.g. methylated BSA in complete Freund's adjuvant [mBSA]) and thereafter face re challenge with intrarticular injection of the same antigen(621;622).

The induction of arthritis is T cell dependent, B cell activation is not considered to be involved in the generation of the hypersensitivity response of this model. CD4+ cell depletion inhibits both the acute reaction, and severity of the chronic disease(623). Antigen induced arthritis is transferred into SCID mice by CD4+ cells and not CD8+ cells(624).

RA is characterised by phases of disease flare. This can be mimicked in the antigen induced arthritis. During the chronic phase, if mBSA is injected intravenously, an exudative flare reaction in the affected joint is induced, that is abrogated by T cell depletion, and not complement depletion, confirming the T cell dependence of the immune compartment (as opposed to erosive compartment) of the disease(625). In addition, re-challenge into the knee around one month after primary challenge elicits the formation of lymphoid aggregates similar to those found in RA(626). Many of these possess discrete T and B cell zones and further CXCR5-/- mice possess reduced erosive disease(626).

K/BxN serum transfer model

A major problem with the transgenic model has been the limited availability of the mice. However, what has been most useful is that serum from KRNxNOD offspring can transfer disease to a wide panel of inbred-strains and gene-disrupted mice with the same onset of arthritic features being observed in almost 100% of mice, in a fashion similar to that seen with the transfer of arthritis by anti-CII antibodies. Whilst a single i.p. injection of antibodies confers a transient arthritis related to the level of residual circulating antibodies, multiple administrations confer an erosive arthritis similar to that seen in the KRNxNOD mice. The antibodies appear to be of the IgG1 (Th2) subclass, with indications that they are closely related and thus derived from a narrow range of B-cells(627;628). Like the passive CII model, except for the fact that CIA antibodies are of the Th1 IgG2a and IgG2b class, mixtures of antibodies derived from serum are required though an arthritis-inducing monoclonal anti-body has been developed(627).

The SCID-human RA mouse model (SCID-HuRA)

SCID (severe combined immune deficiency) mice are homozygous for the spontaneous mutation *Prkdc*^{scid} and are characterized by an absence of functional T cells and B cells, lymphopenia, hypogammaglobulinemia and a normal hematopoietic microenvironment. They were first discovered in 1983 and found to carry a DNA repair defect and a defect in the rearrangement of genes that code for antigen-specific receptors on lymphocytes. Most homozygotes have no detectable IgM, IgG1, IgG2a, IgG2b, IgG3, or IgA. Thymus, lymph nodes, and splenic follicles are virtually devoid of lymphocytes. These characteristics make SCID mice ideal for transplantation of human tissue that can then be biologically manipulated in an environment devoid of immunological interference from the

host. Their discovery was followed by a number of experiments in which tissues such as human haematopoietic stem cells(629) and human thymus(630) were successfully transplanted into SCID mice without subsequent rejection.

The first full report to describe the use of the SCID-HuRA was made in 1993(631) and was of particular importance to research as although a number of animal models of arthritis had been developed none was capable of recapitulating the conditions found within the synovium of RA patients. This report demonstrated that when both normal and inflamed RA synovial tissue was transplanted under the renal capsule of C.B 17 SCID, mice tissues were noted to survive and maintain phenotypic and histological characteristics of pre transplanted tissue albeit with a reduction in T cell numbers. Further reports demonstrated that engraftment of human RA synovium into the knee joint of SCID mice was able to elicit an arthritis(632) and also that synovium transplanted at this site has the capacity to destroy cartilage(633).

The use of the SCID mouse was limited however by the presence of natural killer cells, macrophages and granulocytes, none of which are affected by the SCID mutation and which led to low levels of engraftment, amongst other problems. However, the beige mutation (Lyst bg) in mice is associated with neutropenia, lack of NK cytotoxic activity and lack of functional macrophages(634) and so SCID and beige mice were subsequently backcrossed and a SCID/beige on a C. 17B background was developed.

Thus the immune deficient status of SCID mice can be taken advantage of to transplant pieces of human rheumatoid synovium and investigate their function *in vivo* without the accompanying problems associated with *ex vivo* organ culture, such as artificial growth medium and tissue necrosis through poor oxygen

perfusion and nutrient diffusion(635;636). After transplantation, the human synovial vasculature anastamoses with the mouse via a fibrous capsule and the tissue becomes perfused with the host blood(637). The grafts remain stable for over a month and often longer before starting to slowly lose their architecture. Some grafts appear to recruit murine monocytes or T-cells over time to a minor degree, whilst others do not. Immuno-fluorescence assays of human cell markers in the main do not detect human cells in host mouse lymphoid organs after transplant. Some report that grafts become depleted of T-lymphocytes(637-640) which could indicate turnover of T cells in the diseased tissue, and others that CD3 cells remain after engraftment(641). At 4 weeks cells still express Ki-67 or PCNA, markers of cell division, and the grafts continue to release human immunoglobulins and IL6 for example(641), all of which can be detected in the circulating blood. PCNA is associated with the proliferating αvβ3 angiogenic blood vessels, and proliferating fibroblasts. CD68 macrophages start with a concentration at the synovial lining layer, which becomes less demarcated and distribution more diffuse. CD3 cells can congregate around blood vessels. Fibroblasts become denser with time, and the tissue become more densely packed and organised unlike the looser less organised tissue at implantation. Engrafted pannus at four weeks contains cells expressing a variety of markers such as those indicating helper cells (CD4), fewer T-suppressor cells (CD8 subsets), and B-cells (CD20). B-cell aggregates are maintained throughout, although become less organised(641). Cytokines are synthesised, IL-6, TNFα for example, though IL-6 synthesis can wane unless stimulated by a further stimulus or recruitment of T-cells(637;638). Enzymes indicating the graft's potential to degrade bone and cartilage, such as tartrate-resistant acid phosphatise positive cells (osteoclasts) and

matrix metalloproteinases (MMPs) including MMP1, 3 and 9, are found to be expressed within grafts(642).

Once the vascular anastamoses are established (3 weeks) human lymphocytes can be injected i.v. and their accumulation within the graft assessed; either through labelling with a tracer (e.g. technetium, indium) or via histology using human specific cell markers. Isolated blood lymphocytes, monocytes or T-cells can be studied and U937 cells, a human myeloid precursor cell line, can also be used. Care should be taken if specific RA tissue homing is being investigated since it can be shown that human cells can traffic to other inflammatory tissues as well, showing certain systems are a general feature of the tissue inflammatory state. To control for cell trafficking under non-inflamed conditions, human skin can be transplanted as a control in the same mice.

Adhesion molecule expression changes over time. VCAM-1 and E-Selectin expression decreases with time, whilst ICAM-1 is maintained. The injection of TNFα (1000U) into the graft significantly up regulates ICAM-1, and this is accompanied by an anti-ICAM-1 inhibitable enhancement of human mononuclear cell recruitment into the grafts after i.v. infusion(637). The anti-inflammatory cytokine IL-10 reduces ICAM-1 expression and its partner IL-4 a mild increase in ICAM-1. IL-10 and IL-4 reduce human peripheral blood mononuclear cell recruitment by the grafts(643). These are important observations, establishing the importance of ICAM-1 in the recruitment of mononuclear cells into human RA synovium.

The SCID-HuRA mouse model allows a number of important research applications in RA (Table 4) including the *ex vivo* testing of biological agents(644), the development of synovial specific compounds(645), as well as an

increased understanding of the pathogenic role of specific cytokine and chemokine pathways in RA synovium(339;637). Recently, the presence of human autoantibodies has also been detected in the sera of HuRA-SCID mice(418), raising the possibility that these antibodies are manufactured directly within the synovium. As has been previously discussed and is of particular interest, the synovium has long been recognised to contain sites of ectopic lymphoneogenesis. Whether these sites are truly functional and involved in the production of pathogenic antibodies has caused ongoing debate in the literature(416;426).

When using this system to assess the efficacy of human specific biologics, one end point can be tissue size - anti-human IL-6 receptor antibodies reduce graft size by half, whilst auranofin (an oral disease modifying anti-rheumatic drug) treatment does not(642). Anti-IL-6 reduces inflammatory cell involvement, and the grafts are replaced by fibrous and adipose tissue. MMP1 and MMP9 expression is markedly reduced(642). This would indicate that the erosive potential of the tissues would be reduced. The erosiveness of the RA synovium can be assessed either through the determination of metalloproteinase expression, or implantation in juxtaposition with cartilage(640;646). Synoviocytes can be seen to adhere and erode into the matrices. In the case of human cartilage co-implants, tissue can be seen to burrow into the matrix creating furrows and tunnels. The implants can be graded according to their erosive capacity, and will depend on the donor tissues. Normal donor tissue is not erosive. The cell types associated with this erosion appears to be fibroblastic (type B synoviocytes), and some macrophages (type A) with little contribution by lymphocytes.

Selective depletion and reconstitution experiments have also provided very useful information. The depletion of T-cells with anti-CD2 for example results in not

only an almost total suppression of TNFα, IL-1β, IFNγ, and IL-15 mRNA expression, but also the expression of MMPs. In addition to this, CD68 stained cells also disappear. The administration of autologous CD4 T cells restores TNFα synthesis, stimulates IFNγ synthesis and maintains CD68 cells. IFNγ will replace the role of CD4 T-cells in anti-CD2 treated mice(647). Non-cytotoxic CD8 suppressor T-cells cells (lacking CD40L, the cytotoxic molecules perforin and Granzyme-A) derived from autologous RA synovium have the opposite effect. These cells are seen as closely associated with lymphoid aggregates and their selective depletion results in disaggregation of the germinal centres, an inability to retain dendritic cells and reduced IgG synthesis by the grafts. The depletion of B-cells from synovial tissues containing B-cell germinal centres, results in dissociation of the aggregates, loss of CD4 T –cells, and reduced inflammatory cells, IFNγ and IL-1B(543). Micro-dissection of follicle derived CD4 cells followed by expansion and injection stimulates graft IFNγ, TNFα and IL-1β synthesis(543).

The active angiogenesis within the grafts enables research into this area. The grafts express vascular endothelial growth factor (VEGF) and angiopoitein-1, both potent angiogenic factors. The administration of anti angiogenic factors such as endostatin and TNP-470 (aka AGM-1470) reduce blood vessel density and the retention of inflammatory cell infiltrates. Cell apoptosis is also induced within the grafts(648;649).

Prospective mining of novel targets in the synovial structures is also enabled through this model. This is exemplified by the screening of bacteriophage for adhesion to synovial structures *in vivo* in the transplanted mice(645). This approach has resulted in the identification of novel and selective molecular

structures within RA synovium that can be selectively ligated by novel peptides and have the potential for drug targeting. Genetic manipulation of cells derived from RA synovium can be investigated, one such example are RA synovial fibroblasts, whereby manipulation of MMP, cytokine, cell signalling pathway, expression has been manipulated to provide detailed knowledge of the function of these cells in cartilage erosion *in vivo* (reviewed in (650)).

Table 4: The use of the SCID-human RA mouse model (SCID-HuRA)

Mechanisms in the maintenance of the rheumatoid structure

Synthesis of human RF and ACPA antibodies.

Human lymphocyte migration into the graft: adhesion molecules, chemokines

Human lymphocyte subsets, cloned, or transgenic cell function

Erosive capacity and mechanisms

Human cytokine and chemokine synthesis and actions

Human rheumatoid gene expression

Human rheumatoid gene expression under resting and investigational conditions

Assessment of human species specific biologics

Determination of the translational utility of experimental therapeutics in a human in vivo system

Comparisons between OA, RA, and other human inflammatory conditions

Drug delivery to human RA synovium

Summary and hypotheses

RA is the commonest inflammatory arthritis with associated major health economic implications. At present - despite the revolution that has followed the introduction of biological agents - a number of challenges still face clinicians treating the disease. Importantly, at present it is difficult to predict those patients early on within a defined window of opportunity who have the worst prognosis and will progress to suffering the greatest erosive burden and associated morbidity. Thus refined prognostic markers are required and evidence suggests that these biomarkers may lie within the synovial membrane, the primary site of inflammation in RA. In particular the presence of ELN within the synovial membrane has been suggested to be associated with clinical phenotypes, but its predictive power in a prospective study has not been explored. In addition a robust quantitative means to assess the degree of organisation (and hence ectopic lymphoneogenesis) within the synovial membrane has not been reported.

The prognostic role of synovial ELN is tightly linked to a suggested role for these structures in RA pathogenesis and this concept has generated much debate in the literature. Crucial questions to address are whether ectopic GCs are merely the by-product of inflammation, whether they are capable of functionality and in particular are they involved in the local production of autoantibodies?

Thus the central hypothesis of this thesis is that ELN within the synovial membrane:

1. Identifies specific RA clinical phenotypes
2. Is functional and involved in RA pathogenesis via the local production of autoantibodies

Specific aims and objectives

The specific aims and objectives of this thesis were therefore to:

1. a) Develop and validate a pathological scoring system capable of quantifying the degree of aggregation within the synovial membrane, b) Determine whether the degree of aggregation associates with certain key pathological and molecular markers within the synovial membrane, c) Determine whether the degree of cellular organization within the RA synovial membrane associates with specific clinical phenotypes and, d) Determine whether in established disease the degree of aggregation within the synovial membrane predicts joint damage progression.

2. Determine whether ectopic lymphoid structures in RA synovium are functional by determining whether they: a) Express AID, the enzyme required for SHM and CSR of Ig genes; b) Support ongoing CSR and the production of high-affinity ACPA; and c) Remain functional and promote B cell survival, proliferation, and autoantibody production in a RA/SCID chimera model devoid of any new immune cell influx into the synovium and where the ongoing immunological activity of secondary lymphoid organs is excluded.

3. To elucidate further the functionality of ectopic GCs by determining, a) Whether a population of large CD20+, IgD- B cells surrounding ectopic GCs represent the novel population of large interfollicular B cells, recently described surrounding GCs in secondary lymphoid organs and b) Whether extrafollicular AID expression within IF large B cells within lymph nodes and within the RA synovial membrane is associated with a somatically mutated IgH.

Chapter 2: Materials and Methods

General Protocols

The general protocol sections reported in this chapter (Immunohistochemistry, Quantitative Taqman real-time PCR and Western Blot) describe procedures that have been specifically optimised for work presented in this thesis and are included in the standard operating protocol of the lab. Relevant modifications of these protocols and additional protocols specific for each chapter are reported within the dedicated materials and methods sections of each chapter.

Immunohistochemistry

All materials were purchased from Dako (Glostrup, Denmark), Sigma Aldrich (Poole, UK) and VWR (Poole, UK) unless otherwise stated.

Sample fixation and embedding

Fixation is required to stabilize cells and tissues, therefore making the Ag insoluble (and thus detectable) and protecting the tissue from processing and osmotic damage caused by solutions used during the staining procedures.

All paraffin samples were fixed with 4% formalin or paraformaldehyde (aldehyde-based fixatives) for 24-48 hours prior to embedding.

Frozen samples were fixed prior to staining with cold acetone (-20°C for 10 minutes), paraformaldehyde (4°C for 10 minutes) or 100% ethanol (room temperature) (Appendix I).

Acetone, ethanol and aldehyde based fixatives were both used in this study. Acetone and ethanol fix the tissue by coagulating proteins and inducing denaturation. Conversely, paraformaldehyde and formalin induce the formation of cross links between proteins, making them insoluble.

Paraffin embedding

The process of formalin fixation followed by paraffin embedding allows tissue to be fixed and stored with good preservation of both tissue architecture and cell morphology for prolonged periods of time.

Following dissection, fresh tissue was immediately fixed in 4% paraformaldehyde (Appendix I) for 24 hours at room temperature. The tissue was then placed into embedding cassettes and processed as follows:

1) 70% Ethanol, two changes, 1 hour each

2) 80% Ethanol, two changes, 1 hour each

3) 95% Ethanol, two changes, 1 hour each

4) 100% Ethanol, three changes, 1 hour each

5) Xylene, three changes, 1 hour each

6) Paraffin wax (56-58 C), two changes, 1 ½ hours each

Finally, the tissue sample was embedded into paraffin blocks and place on a cold plate until completely cooled and set (approximately 20 mins).

Paraffin embedded samples were stored in boxes at room temperature until use.

Frozen sample embedding

Fresh tissue was dissected and a drop of O.C.T. (optimal cutting temperature) was placed on the top of a small piece of labelled cork. More O.C.T. was placed on the sample to cover the tissue completely. A small plastic beaker filled with isopentane (2-methyl butane) was placed in liquid nitrogen to bring the isopentane to its freezing point (-160°C). The cork with the embedded sample was then placed in the ice-cold isopentane and left in the solution until completely frozen (O.C.T.

turned white). The sample was then wrapped in aluminium foil, placed in a falcon tube and stored immediately at -80°C freezer until use.

Sample cutting

Paraffin embedded samples

Human tissue samples were placed in boxes filled with ice to cool down for 1 hour prior to cutting. Sequential sections were cut at a thickness of 3µm on a Leica microtome (Knowlhill, UK). The sections were then floated on a warm water bath (45°C), before being picked up onto Superfrost Plus slides (sequentially numbered) and excess water allowed to drain from the slides before being placed on a 70°C hot plate for 1 hour. The slides were then removed from the hot plate and stored in cardboard boxes at room temperature until use.

Frozen samples

5µm sequentially cut sections were obtained by cutting the specimens in a cryostat (Leika) and mounting the tissue onto glass slides (SuperFrost Plus). Slides were left overnight at room temperature to dry. Subsequently each slide was individually wrapped in aluminium foil, labelled and placed in boxes stored at -80°C until future use. Prior to use for immunohistochemical experiments each slide was defrosted for 10 mins at room temperature before being unwrapped and fixed.

Deparaffinisation and rehydration of paraffin embedded tissues

The use of paraffin embedded samples allows the identification of the Ag of interest within the context of a preserved histological structure. However, a rehydration process is necessary in order to permeabilise the tissue before staining.

Before each immunohistochemical experiment (including Haematoxylin and Eosin staining), paraffin embedded tissues were mounted onto slides and treated according to the following protocol.

Slides were placed in a slide holder (glass or metal) and incubated in xylene (2 to 3 changes, 5 minutes each), followed by passages in 100% absolute ethanol (2 changes, 5 minutes each), 70% ethanol (2 changes, 5 minutes each) and 50% ethanol (2 changes, 5 minutes each). Slides were then immersed in distilled water for 5 minutes and placed in jar containing Tris buffer saline (TBS) (Appendix I) until use.

Note: frozen tissue mounted onto slides does not need to be rehydrated before use.

Haematoxylin and Eosin (H&E) staining

In order to accurately visualize the microscopic structure of the specific tissue under study, H&E staining was performed on all tissue samples.

H&E staining, by the use of two diverse dyes, permits the visualization and differentiation of the cellular nuclei and cytoplasm under light microscopy. Haematoxylin stains the chromatin within the nucleus with a deep purplish-blue colour, while Eosin stains the cytoplasmic material, connective tissue and collagen with a pink colour.

Frozen or paraffin sections (following rehydration) were placed in jars containing filtered Mayer's Haematoxylin for 5 minutes. Slides were then rinsed in tap water and placed in 1% Acid Alcohol (Appendix I) for a few seconds. The acid alcohol passage removes the excess of Haematoxylin from the cytoplasm thus allowing Haematoxylin to stain only within the nuclei. The slides were then washed again in tap

water, dehydrated and mounted in an organic solvent soluble mounting medium (DePeX).

Toludine Blue staining

Toludine blue is a basic dye that stains nuclear material blue and sulphated polysaccharides purple, it facilitates sharp structural detail.

Following rehydration, paraffin sections were placed in jars containing Toludine blue solution for 2 mins, rinsed in tap water , dehydrated and mounted in an organic solvent mounting medium (DePeX).

Ag retrieval

The use of formalin (aldehyde-based fixative) during the fixation process of paraffin embedded tissue causes the formation of methylene bridges (inter- and intra-molecular bridges), which cross link proteins in the embedded samples. Formalin-fixed tissue sections therefore require treatment with an Ag retrieval step prior to immunohistochemical staining in order to open free sites for the antigen/antibody binding.

Two methods of Ag retrieval are used within subsequent experiments described within this thesis: 1. proteolytic-induced antigen retrieval, and 2. heat induced antigen retrieval. In some staining experiments requiring the identification of two Ags, double retrieval was performed with both Ag retrieval methods.

1. Proteolytic induced Ag Retrieval

The protein cross-links formed during the fixation of formalin-fixed paraffin embedded samples can be partially broken by the use of proteolytic enzymes (Trypsin, Proteinase K, Pepsin). During the procedure some of the Ags (and indeed overall tissue morphology) may be altered or destroyed by the use of

enzymes and in particular, false binding sites may be created due to excessive tissue digestion. It is important therefore to accurately time (to seconds) the incubation of the tissue with the enzyme.

The enzyme used for Ag retrieval of specimens within this thesis was proteinase K. Prior to use, proteinase K was warmed up to 37°C for 15 min (in order to achieve the optimal enzymatic activity) and placed on the tissue sections on the slides for 5 minutes. After incubation, slides were carefully rinsed in TBS and then immersed in fresh TBS until staining.

Heat induced Ag Retrieval

Heat induced Ag retrieval uses solutions of variable pH (pH 6 or pH 9) to remove the weaker binding formed during fixation (Schiff bases). Heat mediated antigen retrieval techniques can involve the use of microwave oven irradiation, pressure cooker heating, autoclave heating, water bath heating, steamer heating, or incubator.

For immunohistochemical experiments within this thesis I used a water bath to heat Dako Target retrieving Solution in plastic jars to a temperature of 96^0C, requiring a water bath temperature of between 95–99°C. Following deparaffinisation and rehydration steps, slides were immersed in preheated target retrieval solution and placed in the water bath and incubated for 30–50 minutes (depending on the Ag). After incubation, slides were kept in the jars and target retrieval solution but removed from the water bath and allowed to cool down for at least 20 minutes at room temperature. Importantly the target retrieval solution turned opaque on heating and was allowed to return to its original clear colour during cooling. After retrieval slides were rinsed in TBS and left in TBS until use.

Immunohistochemical detection of human Ags

Immunohistochemistry allows the localization of a specific Ag/s within tissue sections. This system is based on the recognition of a specific Ag/s by a conjugated (direct method) or un-conjugated (indirect method) primary antibody (first layer).

The first layer is followed by one or more incubations with a secondary antibody linked to enzymes (commonly horseradish peroxidase HRP or alkaline phosphatase AP) or a biotinylated antibody followed by enzyme-linked Avidin complexes. The use of a substrate solution and a chromogen, while reacting with the used enzyme, allows the precipitation of the colour (colorimetric methods) on the site of antibody-antigen binding.

Colorimetric methods: horseradish peroxidise and alkaline phosphatase

The two more diffuse colorimetric methods used in immunohistochemistry are based on the activity of two enzymes, horseradish peroxidase (HRP) and alkaline phosphatase (AP). HRP reacts with chromogen substrates (3,3'-diaminobenzidine or DAB and 3-Amino 9-ethylcarbazole or AEC). Immunohistohemical experiments described within this thesis use DAB that develops as a brown product, insoluble in alcohol and other organic solvents. Since endogenous peroxidase activity is present in many tissues (including lymph node and tonsil) and can be non-specifically detected with the DAB substrate, pre-treatment of the tissue section with hydrogen peroxide or blocking solution was performed prior to incubation of primary antibody in order to avoid non specific staining. The synovial membrane however lacks endogenous peroxidase activity and thus a blocking step was not required on synovial tissue sections.

AP catalyzes the hydrolysis of phosphate–containing products in the basic pH range. Its enzymatic activity is visualized by using a pink or blue chromogen in an alkaline buffer. The chromogen can be either soluble in organic solvent or permanent. Endogenous alkaline phosphatase is diffusely expressed in human tissue, therefore the inhibition of the endogenous enzyme is mandatory in order to avoid unspecific staining in the section.

Within experiments described in this thesis the chromogen was Vector Red (Vector Lab) and was used according to the manufacturer's instruction (Appendix I). In addition, Levamisole was added to the substrate solution in order to inhibit endogenous activity within tissue sections of alkaline-phosphatase (Appendix I).

Staining procedures: The Avidin-Biotin Complex (ABC) Method

The Avidin-Biotin Complex (ABC) method is based on the strong affinity that Biotin (a small molecular weight vitamin) displays for Avidin (a large glycoprotein of 68 Kd). Avidin presents four binding sites for biotin providing the backbone for a macromolecular amplification complex. Avidin can be labelled with various enzymes (horseradish peroxidase or alkaline-phosphatase) and fluorochromes, allowing diverse methods of detection to be used. In this study I used the DAKO Avidin-Biotin Complex (ABC) (Appendix I) linked to horseradish peroxidase or alkaline phosphatase. The method requires three layers: the first layer is unlabeled primary antibody, the second layer is the biotinylated secondary antibody and the third layer is the enzyme HRP or AP-Avidin conjugate. The used enzyme is then visualized by the use of the appropriate chromogen and substrate. The experiments described within this thesis use HRP and AP conjugated Avidin-Biotin kits from Dako.

In order to avoid non-specific binding to endogenous biotin (present in diverse tissues) pre-treatment with unconjugated avidin saturated with biotin was performed on each slides stained with the ABC system. Tissue sections underwent blocking of endogenous biotin activity using the Dako Biotin Blocking System. Tissue sections were primarily incubated for 10 minutes with preprepared avidin solution. The slides were then rinsed in TBS (x two 5 mins) and then incubated for a further 10 minutes with preprepared biotin solution. This was followed by a thorough washing of the tissue sections with TBS.

Staining procedures: Dako EnVision System Polymeric Method

This system, in comparison to the ABC method described above has higher sensitivity while reducing the number of the staining layers and thus the overall experimental time. The secondary antibody (anti-mouse or rabbit) is directly linked to a dextran polymer binding a large number of HRP or AP molecules. Therefore, after the first layer of unconjugated primary antibody, the EnVision secondary is applied to the tissue sections and after washing and application of the substrate chromogen, the colour is developed. The EnVision double staining system allows the simultaneous detection of more than one Ag in the same tissue section by using mouse or rabbit primary antibodies.

An indirect immunohistochemical staining method on paraffin embedded samples was mainly used within experiments described within this thesis.

Following tissue fixation and antigen retrieval, a wax circle was drawn around tissue sections using a DAKO Pen in order to inhibit the dispersion of solutions applied to the section and thus subsequent tissue desiccation.

All the incubation steps were performed in humid chambers at room temperature.

All the washes were performed in TBS (1%). All primary antibodies were

unlabelled, diluted in the antibody diluent and incubated for 1 hour unless otherwise stated. Appropriate biotinylated secondary antibodies were used. Single staining was performed either by indirect AP-avidin-biotin complex or by indirect HRP avidin-biotin complex.

Envision AP or HRP labelled polymers were used as secondary reagents for all mouse and rabbit primary antibodies. Double staining was performed using the Envision double staining or combining a single staining with avidin-biotin complex with an Envision single staining.

Colour development for both AP and HRP was performed under direct light microscopy and enzymatic reaction blocked as appropriate by immersion in distilled water.

Following immunohistochemical staining of tissue sections using the appropriate substrate for colour development (organic solvent soluble or permanent) the slides were immersed in haematoxylin to define nuclei (10 seconds) and then rinsed in tap water and either mounted with aqueous mounting medium or dehydrated through passages of alcohol and xylene and mounted in a xylene soluble mounting medium (DEPEX).

Antibodies and titration experiments

For primary and secondary antibodies, pilot titration experiments were run to determine the optimal working dilutions on positive controls (human lymphoid tissue). The optimal working dilutions were then used in subsequent experiments. A complete list of primary antibodies, isotype matched controls and secondary antibodies for detection of human targets used in this study is reported within Table 5.

Table 5: Primary and secondary Antibodies used for IHC

Clone Primary Abs	***Specificity***	***Host***	***Source***
L-26	*Human CD20*	*Mouse*	*DAKO*
A2452	*Human CD3*	*Rabbit*	*DAKO*
IF8	*Human CD21*	*Mouse*	*DAKO*
124	*Human Bcl-2*	*Mouse*	*DAKO*
IgD	*Human IgD*	*Rabbit*	*DAKO*
PG-B6p	*Human Bcl-6*	*Mouse*	*DAKO*
EK2-5G9	*Human AID*	*Rat*	*(196)*
M7187	*Human Ki67*	*Mouse*	*DAKO*
MI15	*Human CD138*	*Mouse*	*DAKO*
Secondary Abs			
Rabbit anti-rat biotin	*Rat Ig*	*Rabbit (biotinylated)*	*DAKO*
Goat anti-mouse	*Mouse Ig*	*Goat (biotinylated)*	*DAKO*
Rabbit anti-mouse biotinylated	*Mouse Ig*	*Rabbit (biotinylated)*	*DAKO*
Goat anti-mouse ALEXA 488	*Mouse IgG1*	*Goat*	*Invitrogen*
Goat anti-mouse ALEXA-594	*Mouse $IgG_{2\alpha}$*	*Goat*	*Invitrogen*
Goat anti-rabbit		*Goat*	*DAKO*
ABC-HRP/AP			*DAKO*
Streptavidin Alexa 555	*Biotin*		*Invitrogen*
Envision AP/HRP			*DAKO*

Image capture of tissue sections

Images for IHC were captured using a Nikon digital camera linked to a motorized light microscope (BX60) from Olympus (London, UK). PhotoShop software was used to save and reduce the size of the images.

Quantitative Taqman real-time PCR

Extraction of total RNA from human tissues

RNA was extracted using the Qiagen RNeasy mini kit according to the manufacturer's instructions. Tissues stored at -80°C in RNA Later were defrosted on ice, weighed and then cut in order to obtain approximately 20mg (30mg of tissue is the maximum amount suggested in the protocol to avoid reduction in RNA yield and purity). RNA was also extracted from human lymph node as a positive control.

Tissues were placed in a sterile, RNase free tube and 600µl of RLT buffer (containing denaturing guanidine thiocyanate with the addition of 10µl of fresh β-Mercaptoethanol/ml of RLT buffer) were added. Tissues were homogenised using a rotor–stator homogenizer until the sample was uniformly homogeneous (usually 20-40 sec). Tissue lysates were centrifuged for 3 minutes at maximum speed (13000rpm) in a micro centrifuge and the supernatant transferred to a new RNase free micro centrifuge tube. An equal volume of 70% ethanol was added to the lysate and immediately mixed by pipetting in order to precipitate RNA, which remains in the aqueous phase. An aliquot (700µl) of the sample was added to the RNeasy mini column, placed in a 2 ml collection tube and centrifuged for 15 s at ≥8000 x g (≥10,000 rpm) to allow the RNA to bind to the silica column. The flow-

through was discarded and the remaining sample (approximately 500µl) added to the RNeasy mini column and centrifuged as above.

To avoid any possible DNA contamination, a DNase step was included according to the manufacturer's instructions. 350µl of Buffer RW1 was added to the column, centrifuged for 15 s at ≥8000 x g (≥10,000 rpm) and the flow-through discarded. 10µl of DNase I stock solution (previously prepared by dissolving solid DNase I (1500 Kunitz units) in 550 µl of RNase-free water) was added to 70µl of Buffer RDD, gently mixed and added to the RNeasy mini column silica-gel membrane. Following a 15 minute incubation at room temperature (RT), 350µl of Buffer RW1 was added to the column, centrifuged for 15 s at ≥8000 x g (≥10,000 rpm) and the flow-through discarded.

The RNeasy column was transferred into a new 2 ml RNase free collection tube. In order to wash away contaminants (residual DNA and protein) in the organic phase, 500 µl of Buffer RPE was pipetted onto the RNeasy column and centrifuged for 15 s at ≥8000 x g (≥10,000 rpm) to wash the column. The flow-through was then discarded and another 500 µl Buffer RPE added to the RNeasy column. The tube was centrifuged for 2 min at ≥8000 x g (≥10,000 rpm) to dry the RNeasy silica-gel membrane and the flow-through discarded. To eliminate any chance of possible Buffer RPE carryover the tube was centrifuged again for 1 min at full speed (13,000 rpm).

For elution, the RNeasy column was transferred to a new RNase free 1.5 ml collection tube, and 30 µl of RNase-free water was pipetted directly onto the RNeasy silica-gel membrane and the tube centrifuged for 1 min at ≥8000 x g (≥10,000 rpm). The elution was immediately frozen at -80°C until required.

Quantification of total RNA

Total RNA isolated within each sample was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer. This instrument allows for the accurate quantification of RNA held within 1µl of solution and eliminates the need to waste large volumes of precious RNA in traditional cuvettes used in standard spectrophotometers. The principle behind RNA quantification using NanoDrop technology is based on quantification of UV absorbance by nucleic acids, namely the Beer-Lambert law. This law predicts a linear change in absorbance with concentration of nucleic acids. Absorbance is measured at both 260 and 280 nm. An absorbance at 260nm of 1 is equivalent to approximately 40µg/ml of RNA and as such this reading is used to determine RNA concentration in solution. As RNA has its maximum absorption at 260nm, a further reading at 280nm is used to assess purity of RNA in solution. This is determined by the knowledge that pure RNA has an A260/A280 of 2.1, thus a value between 1.8-2.0 indicates a relatively pure RNA sample although this result is dependent somewhat on the possible contaminants.

1-2µl of sample is pipetted directly onto the measurement pedestal and surface tension holds the sample in place whilst measurement is made, which takes less than 10 seconds. The spectrum and its analysis is then displayed on the screen of the attached personal computer. The Nanodrop provides a scan of the absorbance of sample from about 200nm up to 350nm, the relevant range for determining RNA concentration and purity.

Determination of RNA quality and integrity

RNA integrity was assessed by resolving total RNA on a 0.8% agarose gel. Agarose powder, in 1x Tris Acetic Acid EDTA (TAE) buffer (see Appendix II)

was boiled in a microwave for 2 minutes, then ethidium bromide (a fluorescent dye that binds to nucleic acids) was added and the solution poured into a minigel tray where a well-comb was inserted. After polymerization, the comb was removed and the gel was placed in a minitank and covered with 1x TAE running buffer (Appendix II).

Samples were mixed with blue loading dye (1:6) to monitor migration, loaded into the gel wells and run at 80V (voltage) for 30 minutes. RNAs were visualized by fluorescence of the incorporated ethidium bromide using a UV light transilluminator and examined for RNA degradation and DNA contamination.

RNA should demonstrate two bands on electrophoresis (corresponding to the 28S and 18S of eukaryotic ribosomal RNA), with the intensity of the 28S band being approximately double that of the 18S band. If the 18S band appears more intense than 28S band, or a smear is present, RNA degradation is likely. Extra bands at high molecular weight normally indicate genomic DNA carry-over.

Reverse Transcription PCR

In Reverse-Transcription PCR (RT-PCR), mRNA is converted to complementary DNA (cDNA) using a reverse transcriptase step before being amplified. RNA was reverse transcribed to cDNA using the Thermoscript RT-PCR System for First-Strand cDNA Synthesis (Invitrogen, cat#11146-016). Briefly, 1μg of extracted total RNA from each sample was mixed with 1 μl of Oligo(dT)20 Primers (50 μM) and 2 μl of 10 mM dNTP mix and brought to a 12 μl volume reaction with DEPC-treated water in a 0.5ml PCR tube. After briefly spinning down in a microcentrifuge, samples were incubated for 5 min at 65°C to denature RNA tertiary structure and samples were immediately cooled on ice to allow RNA and oligos to anneal. For the final reaction, 8 μl of a master mix containing 1 μl of

ThermoScript™ RT (15 U/μl), 1 μl of 0.1 M DTT, 1 μl of RNaseOUT™ Ribonuclease Inhibitor (40 units/μl), 1 μl of DEPC-treated water and 4 μl of 5X cDNA synthesis buffer were added. After mixing and a brief spin down of the tubes, the reverse transcription to cDNA was run in a PCR machine (Applied Biosystems 9700) for 1h at 50°C and the reverse transcriptase was inactivated at 85°C for 5 min. In order to remove the original RNA that could interfere with the quantitative real-time PCR analysis, RNA digestion was performed using 1μl of E. coli RNase H (2 units/μl) at 37°C for 20 min. Finally, the completed cDNA strand was diluted to a final concentration of 10ng/μl with DEPC-treated water and stored until used.

Quantitative Taqman real-time PCR

PCR involves amplification of a specific DNA sequence, which spans between two sequences of primers. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each cycle (in real time) as opposed to the endpoint detection by conventional quantitative PCR methods; it is based on the detection and quantification of a fluorescent reporter whose signal increases in direct proportion to the amount of PCR product in the reaction.

For quantitative Taqman real-time evaluation of mRNA expression levels of human genes, sequence-specific primers and probes from Applied Biosystems were used. Each gene expression assay contains, together with the forward and reverse primers, a Taqman MGB probe with a FAM reporter dye at the 5' end. Within the probe, the dye is linked to a non-fluorescent quencher; during the polymerase reaction, the probe is detached from the cDNA and the quencher is released allowing fluorescence emission from the reporter dye.

The real-time PCRs were run in triplicate on 384-well PCR plates (Applied Biosystems) with an equal loading of 10ng of cDNA/well and detected using the ABI PRISM 7900HT Instrument. The thermal cycling conditions used, comprised a 2 min UNG activation step at 50 ºC, a 95 ºC Taq polymerase enzyme activation step for 10 min, and cycles of 95 ºC denaturation for 15 sec and 60 ºC anneal/extension for 60 sec. Results were then analyzed after 40 cycles of amplification using the ABI PRISM 7900HT Sequence Detection System Version 2.1 (SDS 2.1). In addition cDNA from normal human lymph nodes was used as a calibrator. Relative quantification was measured using the Comparative Ct (Threshold Cycle) Method. Two different endogenous controls (human beta-actin and mammalian 18S) were used to normalize for the cDNA of each sample. The ΔCt for each of the triplicate (Ct of the target gene minus Ct of the endogenous control) and then the average ΔCt of the triplicates were calculated. When a single value within each triplicate differed substantially from the other two values the skewed value was excluded from the analysis. To calculate the □□Ct, the □Ct of each sample was subtracted to the chosen reference sample (usually a RA synovium from a normal individual). The relative quantity was then calculated following the equation RQ= $2^{-\square\square CT}$ where 2 represents doubling of the amount of the product of amplification after each PCR cycle.

Western blotting

Western blotting was used to determine effective citrullination and the specificity of citrullinated fibrinogen for ACPA. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) involves the separation of a mixture of proteins, the blotting of these separated proteins onto a nitrocellulose membrane and their detection using specific antibodies. The polyacrylamide gels

are run in the presence of the anionic detergent SDS that denatures and binds to proteins in a constant-weight ratio leading to identical charge densities for the denatured proteins. Thus, the SDS-protein complexes migrate in the polyacrylamide gel according to size and not charge.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins can be separated based on their molecular weights. They migrate in response to an electrical field through the pores of a SDS polyacrylamide gel matrix toward the anode, under denaturing conditions. A discontinuous buffer system was used, with buffers of different pH and composition to generate a discontinuous pH and voltage gradient in the gel.

Polyacrylamide gels form after polymerization of monomeric acrylamide into polymeric polyacrylamide chains and cross-linking of the chains by N, N'-methylenebisacrilamide. The discontinuous polyacrylamide gel consisted of an upper 4% stacking gel with large pore size where proteins were concentrated and a 10% lower separating gel with smaller pore size where they were separated.

A separating gel mix was carried out using a mini Protean II system (Bio-Rad): in a beaker, 10% acrylamide/bisacrylamide separating gel monomer solution containing 1.5M Tris-HCl and 10% SDS was de-gassed under vacuum for 20 minutes in order to avoid the inhibition of acrylamide polymerisation by oxygen; the polymerisation initiator and enhancer, 10% APS and 0.1% TEMED respectively, were then added and the solution poured between two glass plates separated by 1.5mm spacers. A thin layer of water was placed on top of the solution to exclude oxygen during polymerisation.

After 1hr the layer of water was poured off, a well-forming comb was inserted carefully and a 4% acrylamide/bisacrylamide stacking gel monomer solution

containing 0.5M Tris-HCl, 10% SDS, 10% APS and 0.1% TEMED, was poured on top of the separating gel. The stacking gel was left to polymerise for 1hr, after which the comb was removed and the wells washed twice with electrophoresis running buffer (containing 0.2M Tris base, 1.5M glycine and 10% SDS). The gel was assembled in the electrophoresis tank according to the manufacturer's instructions (Bio-Rad) and electrophoresis running buffer poured into both the inner and outer chambers.

The results of the DC colorimetric protein assay were used to calculate the volumes of samples required to ensure the loading of an equal amount of protein.

Equal amounts of protein samples (usually ~40μg) were denatured by five minutes boiling in 6x loading buffer, containing 0.5M Tris-HCl (pH 7.4), 30% glycerol and 1% bromophenol blue colorant, in the presence of 10% SDS and the reducing agent β-mercaptoethanol.

The protein samples were loaded into the gel lanes and then separated by electrophoresis at 140V for the first 10 minutes and 100V for the subsequent 60 minutes.

A pre-stained molecular weight marker containing labeled proteins of specific molecular weights was run in parallel and used to visualize protein migration and calculate protein molecular weight.

Blotting onto nitrocellulose membrane

Following electrophoresis, proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane, with 0.45μm pore size, using a mini transblot apparatus (Bio-Rad). Membrane, gel, two pieces of filter paper and two fibre pads per gel were soaked in the blotting buffer (containing 1M Tris base, 1M glycine, 10% SDS and 20% methanol) to equilibrate for 10 minutes.

A transfer cassette was assembled consisting of a sandwich containing the following layers; fibre pad, filter paper, gel, membrane, filter paper and fibre pad. The cassette was then placed in the blotting tank according to the manufacturer's instructions, all fully covered with blotting buffer.

Electrophoretic transfer of negatively charged proteins from the gel to the membrane was achieved by the application of a 100V current for 1hr.

Equal protein loading and transfer efficiency to the membrane was verified using the protein dye Ponceau S. The membrane was rocked gently with 0.1% Ponceau S staining solution for 10 minutes and then destained with water until pink protein bands appeared on the membrane.

Enhanced Chemiluminescent (ECL) detection

Immunocomplexed bands were detected by enhanced chemiluminescence according to the manufacturer's protocol (Amersham Life Science).

The method is based on the detection of the emitted light resulting from the dissipation of energy from a substance (in this case, luminol) in an excited state.

Briefly, the HRP enzyme catalyses the oxidation reaction of luminol in the presence of enhancers (phenol). Following oxidation, luminol is in an excited state of energy. During its decay to ground state it emits light, at a maximum wavelength of 428nm, which can be captured on autoradiography film. The intensity of light emitted is proportional to the amount of HRP enzyme and, consequently, to antigen present.

The membrane was incubated with an ECL reagent mix (0.125ml/cm^2 of membrane) containing luminol and enhancers for 1 minute at room temperature. The membrane was then wrapped in Saran Wrap and exposed, under red light, to light-sensitive autoradiography film (Hyperfilm ECL) for various time periods.

Table 6: Genes, specific primers and probes used for QT-PCR

Gene	RefSeq	Gene Expression Assay ID
AID	NM_020661	Hs00221068_m1
CD21L	NM_001006658	Hs01079084_g1
BAFF	NM_006573	Hs00198106_m1
APRIL	NM_003808	Hs00182565_m1
LTβ	NM_002341	Hs 00242737_m1
CXCL13	NM_006419	Hs00757930_m1
TNFα	NM_00594.2	Hs00174128_m1
Human β actin	NM_001101	Hs99999903_m1

Source of all primers and probes: Applied Biosystems.

Single cell laser capture micro dissection (LCM) and amplification of IgH

The technique of LCM allows for the selective adherence of visually targeted cells to a thermoplastic membrane. The equipment for LCM comprises an inverted microscope under the control of a personal computer an infrared laser and control unit, a microscope stage controlled by a joystick and a colour monitor (Figure 15). A cap with the adherent thermoplastic membrane is held by a robotic arm over the tissue section which is mounted onto a membrane covered slide. A laser beam is used to melt the membrane under the tissue section and once adherence of the tissue to the activated membrane within the cap overcomes adherence of the tissue to the slide, selective removal of desired cells is achieved (Figure 15). The cap then fits onto a standard microcentrifuge tube suitable for use in subsequent PCR reactions.

Statistical analysis and Ethics

Statistical methods and ethics approval are discussed within the materials and methods section of each individual chapter.

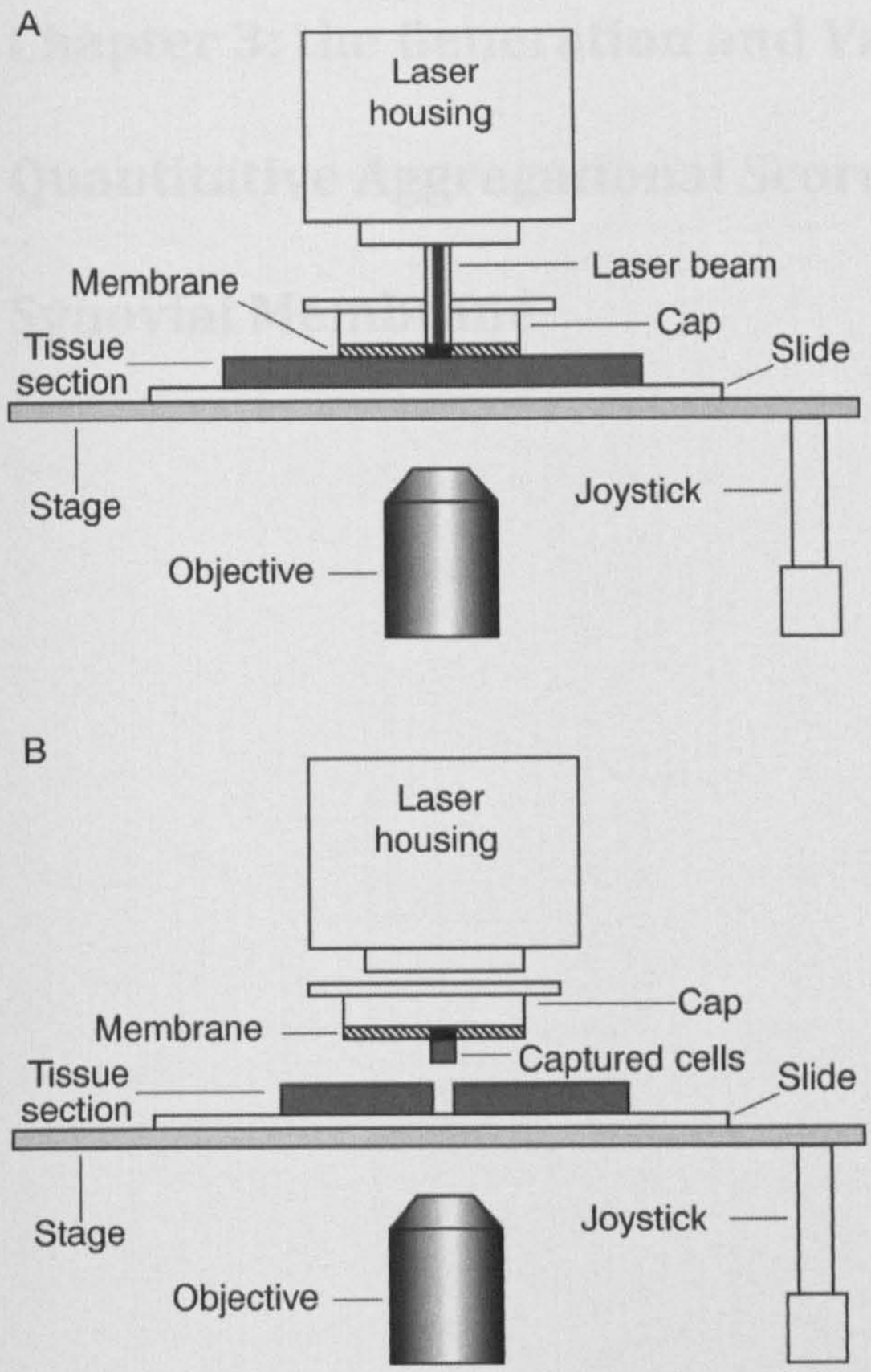


Figure 15: Schematic representation of laser capture microdissection

(A) Activation of the laser leads to focal melting of the polymer membrane. (B) Lifting of the cap selectively detaches the cells adherent to the activated membrane. Reproduced from (3) with permission from the BMJ publishing group Ltd.

Chapter 3: the Generation and Validation of a Novel Quantitative Aggregational Score for the Rheumatoid Synovial Membrane

Introduction

Rheumatoid arthritis (RA) is one of the most important chronic inflammatory disorders in the UK. It affects 1% of adults and causes considerable morbidity, substantially reduces quality of life and has a significant mortality(651) (652;653). It results in large direct medical costs (in the UK, this amounts for more than £600 million (654), with comparable figures reported in the USA(655)) as well as extensive indirect societal costs for the individual and their family and carers.

Treatment of RA remains unsatisfactory despite major advances over the last two decades. Conventional disease modifying anti-rheumatic drugs (DMARDs) such as methotrexate improve symptoms, and reduce disease progression (rather than)but do not prevent the development of erosions(656) (657-659). The concurrent use of combinations of two or more DMARDs results in a greater improvement but still rarely does more than slow the rate of erosive damage(660) (661). Importantly over time it is this erosive damage that accounts for the disability that is seen in RA (662). Recent data have shown for the first time that we are now able to prevent the onset of erosion by use of biological agents such as anti TNF(663) (133;134) early on in disease. Hence expert opinion now favours the use of aggressive treatment (664) during an apparent "window of opportunity" as if this is missed erosive damage is inevitable. Use of these agents is, however, limited by their high costs (approximately £10k/patient/annum) (665). In addition, even in combination with methotrexate 30-40% of patients do not respond to treatment (666). The reasons for this are not currently known, but it is clear that RA as currently classified, encompasses a heterogeneous group of conditions with genetic, biological and clinical variability. This heterogeneity is expressed as a very variable clinical course, with not all patients having a poor outcome and

scores for joint damage varying 10-12 fold after 10 years of disease (26). Recently published data have shown that the heterogeneity seen in radiographic scores appears to be divided into 6 main patterns, with some 20% following a more benign course(667). For these reasons treatment ideally should be tailored on an individual patient basis, maximising treatment to those most likely to have the most severe course, as giving all patients with early RA maximal treatment would not only incur high costs but the substantial risks of side effects would be unacceptable (665;668). There are some well-known markers of poor outcomes, particularly the presence of high levels of auto antibodies including rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) antibodies (669). However these markers are insufficiently sensitive (670) (671) to reliably identify patients within this window of opportunity who go on to have the worst outcomes.

A potential source of prognostic biomarkers is the synovial membrane. Weyand and colleagues(217) have characterised the inflammatory infiltrate into three mutually exclusive patterns: diffuse infiltrate (50%), aggregate with a variable degree of T/B cell segregation (25%) and follicular structures (ectopic lymphoid neogenesis [ELN]) with the formation of germinal centres (GC) (25%). Importantly Weyand has reported that: follicular synovitis is associated with a more severe disease both clinically and radiologically and that each pattern of synovitis is associated with a particular cytokine profile (which may explain the associated degree of erosive damage seen)(421;425). This classification, however, relies on research from a single group and is predominantly inferred from synovium obtained from a small patient cohort during arthroplastic surgery in severely destroyed joints, the histological appearance of which may be different to that in established and early RA(672). Recent reports have also shown that the

synovial membrane has a more continuous spectrum of inflammatory infiltrate without the originally proposed mutually exclusive distinction between grades(334;388), making the application of the Weyand histological subtypes unfeasible. This is emphasised by the variation in diffuse infiltrate seen between synovial tissues with some lacking any infiltrate whilst others having a densely infiltrated, albeit unorganised synovium, both of which would be termed diffuse under the Weyand system, although they may reflect distinct clinical phenotypes. Indeed, a sized based classification system for lymphoid aggregates has been proposed with a division into three grades according to radial cell count, grade 1 (2-5 cells), grade 2 (5-10 cells) and grade 3 (>10 cells) (391). Ectopic GC like structures have been shown to be exclusive to grade 3 aggregates (334) and importantly to be capable of functioning as sites of antigen driven B cell proliferation (415;673). This suggested functionality is further supported by their association with the presence of RF in the serum, suggesting a role for them as ectopic sites of autoantibody manufacture (673).

Thus in order to compare reliably synovial biopsies for both degree of organisation and diffuse infiltrate, both sequentially and between patients, a quantitative pathological scoring system is required. A number of reports have already attempted to link synovial histological scoring systems with clinical phenotype including the semi quantitative Rooney(448) and Koizumi scores (450)and the quantification of sublining macrophage number. The Rooney score does not associate with joint damage progression in established disease(176). The Koizumi score only associates with erosive damage in a cross sectional cohort (451) and reports of macrophage number as a potential biomarker produced conflicting results(454;455).

Further the advent of digital image analysis (DIA), which allows rapid semi automated processing of tissue, has revolutionised the examination of the synovial membrane. Indeed validation of DIA has shown it to be not only equivalent to the gold standard of manual counting (MC) (444) but to minimize bias, to be valid for the widest range of pathological markers and to be more sensitive to changes within the synovial membrane than semi-quantitative analysis (445)and has therefore been suggested to be the optimum tool for pathobiological assessment of the synovial membrane in randomised control trials(674).

Thus there is a pressing need for novel biomarkers in RA and lymphocytic organisation appears to hold potential. It was on this basis that I aimed to develop and validate a quantitative scoring system for rheumatoid synovial tissue, using DIA to accurately quantify both the degree of aggregation and diffuse infiltrate within each tissue sample.

Aims and objectives

The aims of this study were to:

i. Validate the use of DIA within our laboratory as a means to quantify mononuclear cell number within synovial tissue

ii. Develop a quantitative aggregational score (QAS) using DIA to determine sensitively and reliably the degree of lymphocytic aggregation and diffuse infiltration within RA synovial tissue.

iii. Validate the QAS by a comparison with a previously reported system of histological classification (334)

iv. Validate the QAS by determining intra and interobserver reliability

Materials and methods

Patients and samples

Paraffin embedded synovial tissue was collected from 24 RA patients following arthroplastic joint surgery and was used for the generation and validation of the quantitative scoring system. All patients fulfilled the 1987 revised American College Rheumatology (ACR) guidelines for RA. All procedures were performed following written informed consent approved by the hospital's ethics committee (REC 05/Q0702/1).

In addition paraffin embedded synovial tissue was available from 60 patients from the DAMAGE study cohort, described in detail in (176) and, this tissue was used to determine reliability of the histological scoring system developed. All patients fulfilled the 1987 ACR criteria for RA(12). All patients gave their informed consent for biopsy and the study received approval from the Ethics of Human Research Committee of St George Hospital, Sydney, Australia.

Validating Digital Image Analysis to quantify synovial cellular number within our laboratory

Although the validation of DIA has shown it to be equivalent to the gold standard of MC in determining cellular number within synovial tissue(444), it was important to validate the technique within our laboratory. Thus in order to determine whether the application of DIA within our laboratory could reliably determine number of nuclei within a defined area of synovial tissue, 12 RA patients were identified from the synovial biopsy bank and 5μm thick paraffin embedded sections cut from each block and stained with standard H&E. Sections were then digitised to create a stitched together image of the whole section of

tissue using an Olympus microscope fully slaved to an Image analysis programme. 5 random areas within each tissue were then defined using a digitising tablet coupled to the image analysis software and the number of nuclei within each area (region of interest [ROI]) determined using MC. A second independent observer, blinded to initial results, then used the colour threshold technique to discriminate haematoxylin stained nuclei (and hence cellular number) within each defined area. Numbers of nuclei within the defined area were then determined using the image analysis software programme and correlated with numbers of nuclei determined by MC. In addition as nuclei within lymphocytic aggregates were noted to be at a higher density than in intervening tissue areas and thus potentially more difficult to discriminate, aggregates within all tissue sections were defined (Figure 16) and nuclear number (and nuclear area using DIA) within aggregates determined using both MC and DIA.

Determining the effect of different histological staining techniques on the reliability of DIA to determine nuclear number

As DIA uses colour threshold analysis to discriminate nuclei within synovial tissue sections I hypothesised that different tissue staining techniques may have an effect on the accuracy of DIA to determine nuclear number within tissue sections. Three RA patients were identified from the synovial tissue biopsy bank with varying degrees of synovial inflammation and from each patient three sequential 5μm sections of paraffin embedded synovial tissue were stained each with standard H&E, toludine Blue and the B cell marker CD20 as previously described(675) and then developed with alkaline phosphatase and vector red (Vector Red Labs). Three aggregates within each section were then identified and nuclei counted

manually and then quantified using DIA by a second blinded observer. Results from DIA were then correlated with number of nuclei as determined by MC.

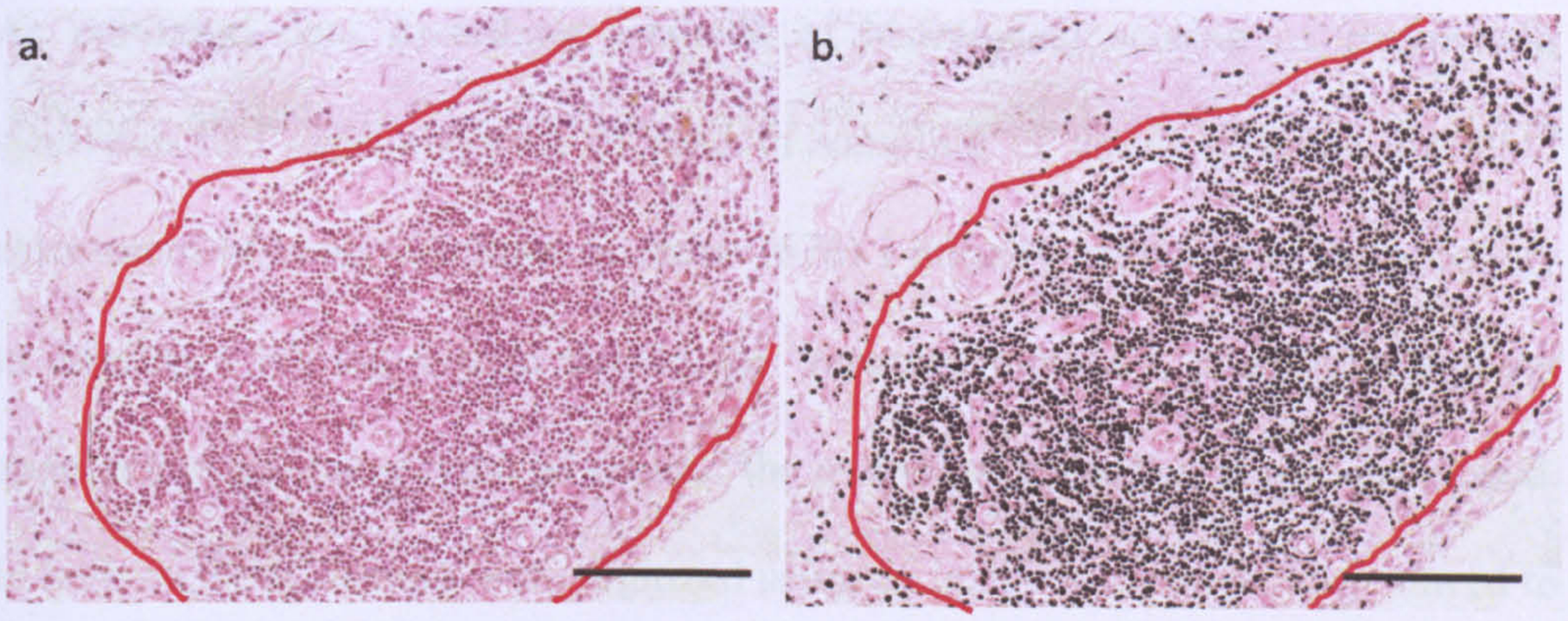


Figure 16: Segmentation of haematoxylin stained nuclei

Using colour threshold analysis haematoxylin stained structures were discriminated within synovial tissue within areas defined by a digitising tablet (outlined in red) coupled to the Image analysis software. Size based filters particles were used to eliminate any small nonnuclear structures. Scale bars represent 200μm.

Development of a quantitative aggregational score for RA synovium

As published data describing methods of histological scoring of rheumatoid synovium had used a system of random sampling of tissue to allow the rapid assessment of large quantities of tissue (676) I initially applied this methodology to develop a scoring system termed the "montage method". In addition a novel approach to assessing synovial tissue, "the overview method" was also developed. In both methods sections were digitized using an image analysis package slaved to a fully motorized Olympus microscope (Figure 17a) and an aggregate was defined as "a clearly demarcated region of high mononuclear cell density, distinct from the surrounding tissue, organized concentrically, perivascularly and greater than 5 cells in diameter".

Quantification of aggregational tendency of synovial tissue using "the montage method"

H&E stained 5μm thick sections of paraffin embedded RA synovial tissue were examined at low power (x4 magnification) and a complete image of each section constructed by automated image capture if identifiable lining layer was present (Figure 18). The complete image of each synovial section was then used to automatically generate a series of systematic uniform random samples of high power fields (hpfs) (x20 magnification) overlying the section representing approximately 20% of the total section area (Figure 18). Any hpfs containing areas of synovium without at least one border of lining layer were excluded from further analysis. A digitizing tablet coupled to the image analysis software was used to outline the area occupied by synovium and mononuclear cell aggregates on each of the selected hpfs. Next, the haematoxylin stained nuclei per field were segmented (separated from other synovial structures) using colour threshold analysis (Figure

16). The area fraction (A/a) of total synovial tissue analysed occupied by mononuclear cells in aggregate form was determined by integrating the regions of interest previously delineated with the nuclear density data provided by threshold analysis. The montage method is summarised in Figure 19.

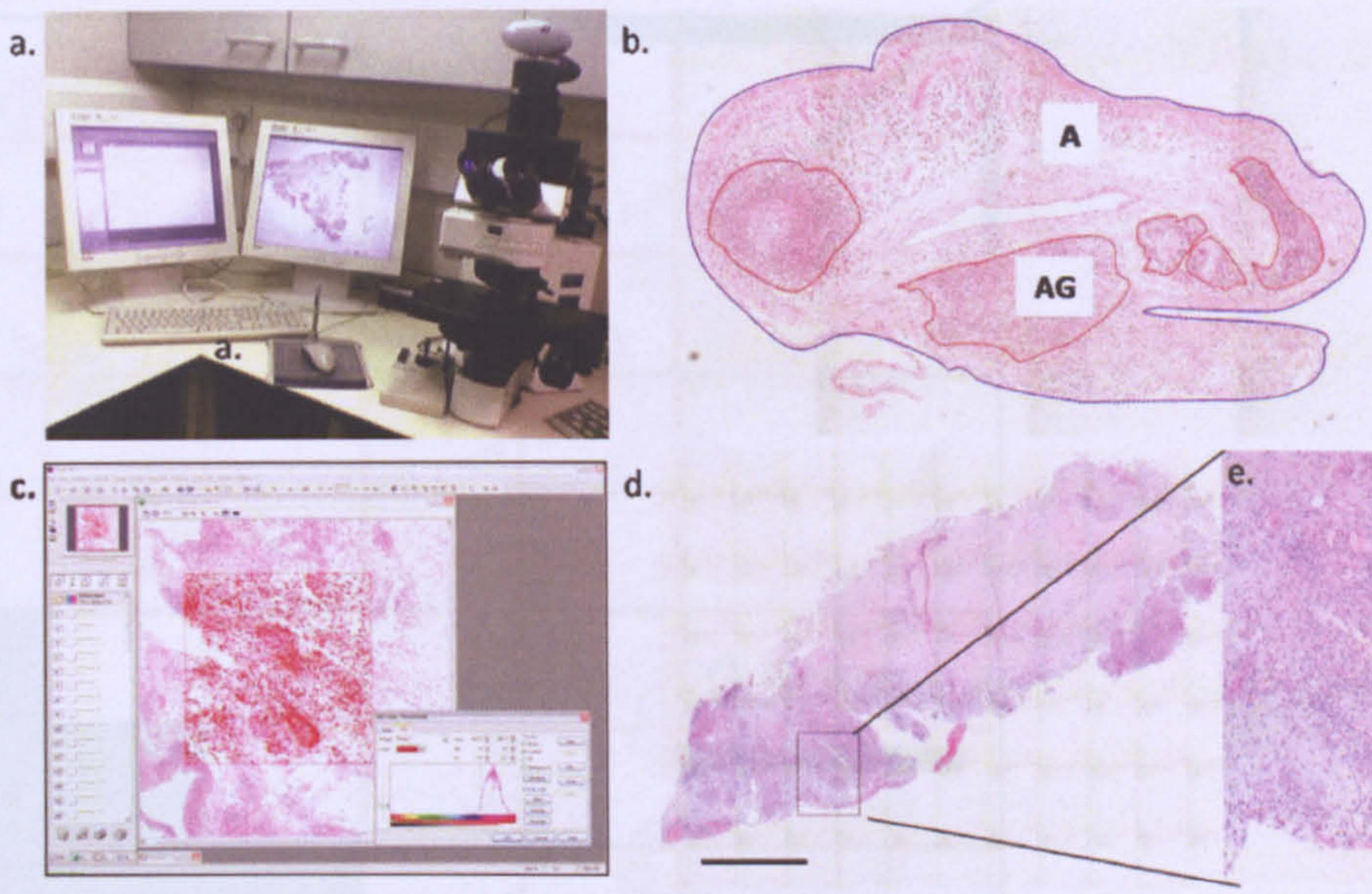


Figure 17: The overview method

1. Sections for both the montage and overview methods were digitised using an image analysis package slaved to a fully motorized Olympus microscope
2. A digitised section of RA synovium. Total area of section (A) is outlined in blue. Aggregate areas (AG) within the section are outlined in red
3. Nuclei within an aggregate area are discriminated using colour thresholding via a histogram
4. The original digitised section can be viewed at higher resolution without loss of quality (e)

Scale bars represent 0.5mm (d) and 30μm (e)

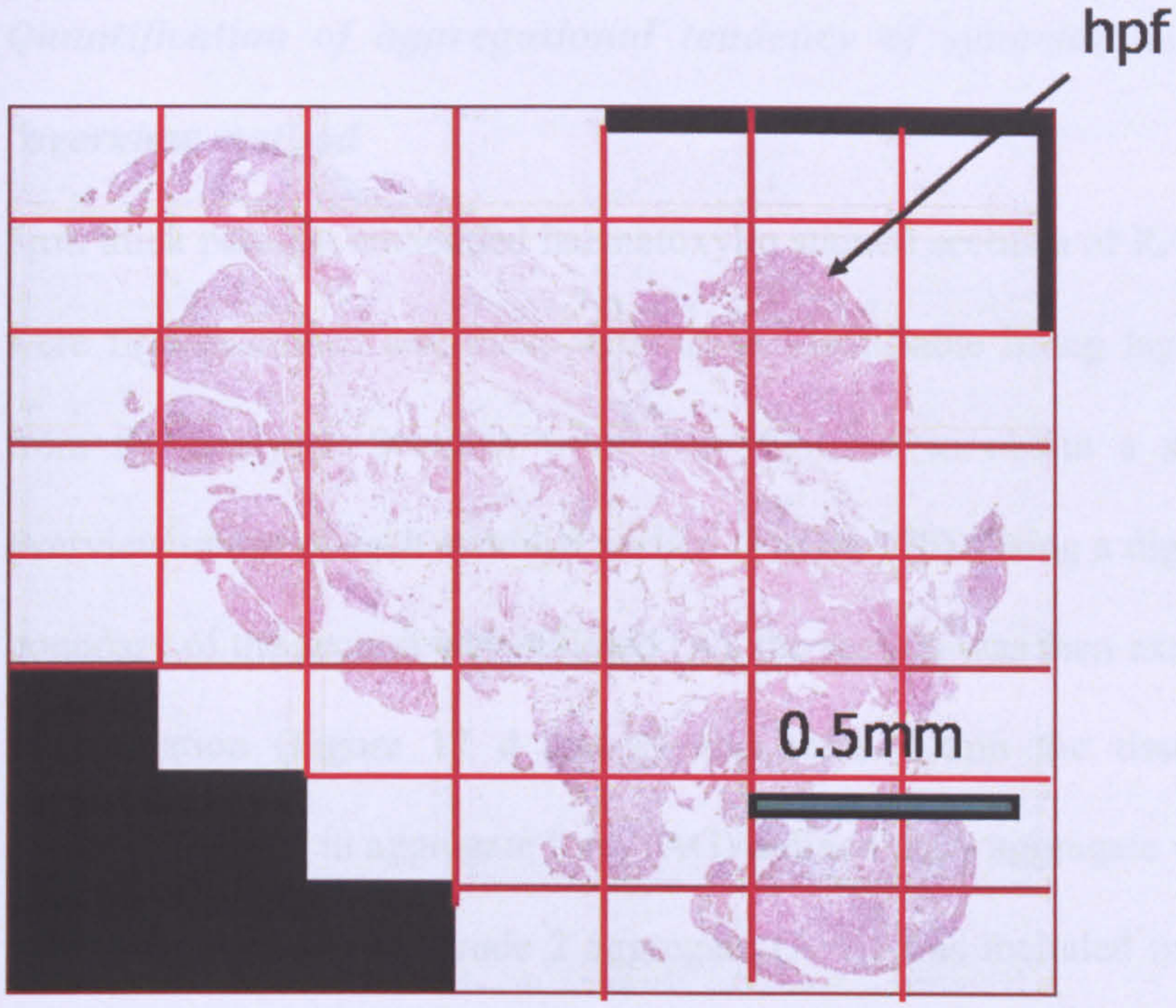


Figure 18: A complete image of each section of synovial tissue constructed by automated image capture "the montage method"

A complete image of each synovial section was generated. Individual squares within the overlying grid represent each individual high power field (hpf). The image analysis software was used to automatically generate a series of systematic uniform random samples of hpfs that were then used for analysis of lymphocytic organisation.

Quantification of aggregational tendency of synovial tissue using the "overview method

5μm thick paraffin embedded haematoxylin stained sections of RA synovial tissue were first examined and those without an identifiable lining layer were omitted from the analysis. Sections were then digitized to obtain a stitched together overview image of each synovial section (Figure 17b). Using a digitizing tablet the boundary of the section was outlined (A), the section was then examined at higher magnification (Figure 17 d and e) and areas within the tissue occupied by mononuclear cell in aggregate form (AG) defined. Any aggregate with a radial cell number greater than 5 (grade 2 aggregate (334)) was included in the analysis. A histogram was then used to discriminate nuclei within the outlined areas (A and AG) (Figure 17b) and to calculate the area occupied by nuclei. This could then be used to calculate the following:

1. The Aggregate Score (AS) = area fraction of whole tissue occupied by mononuclear cells in aggregate form

2. The Diffuse score (DS) = area fraction occupied by mononuclear cells in the remaining tissue.

The overview method is summarised in Figure 20.

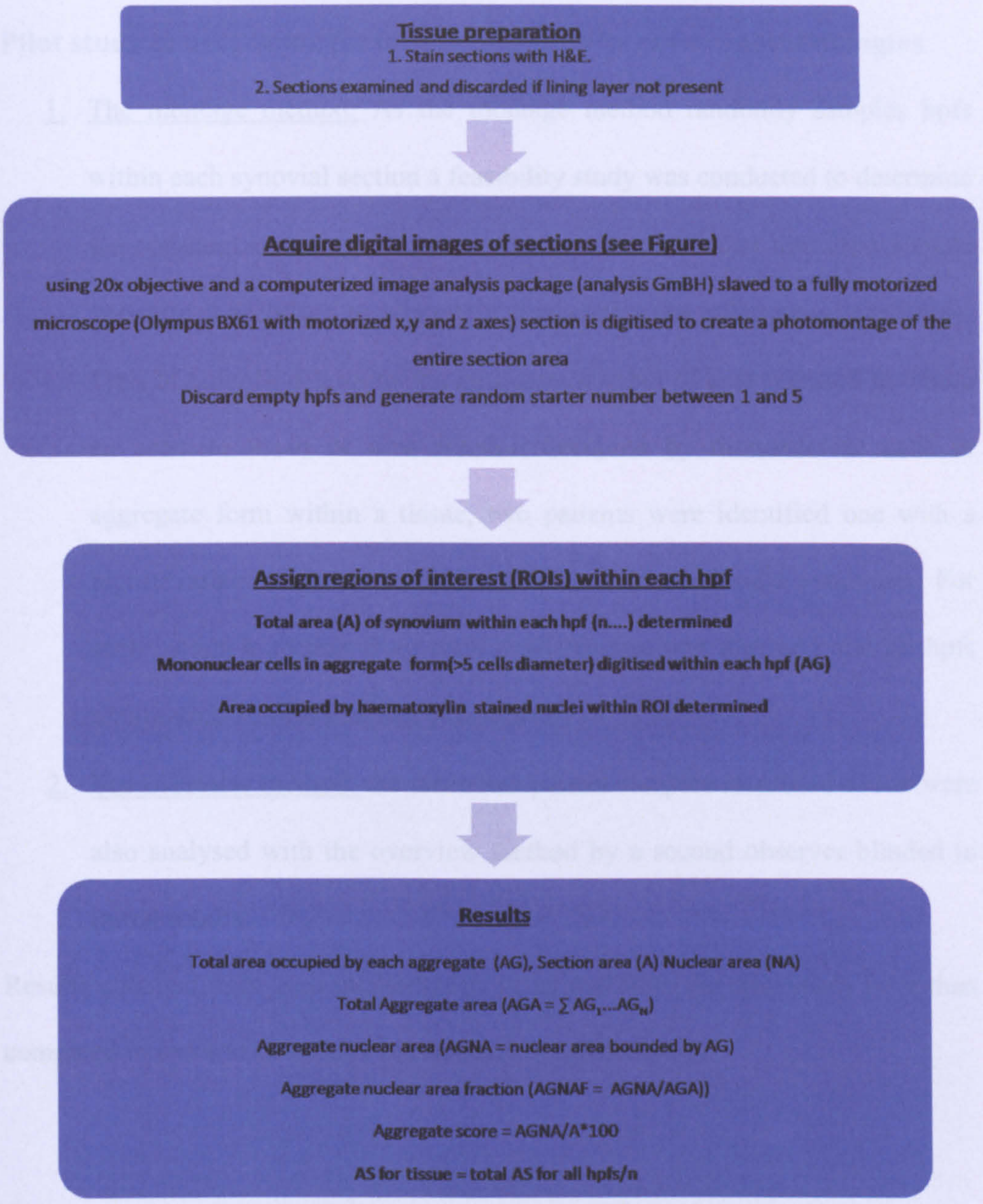


Figure 19: Flow chart illustrative of the montage method for quantifying degree of mononuclear cell aggregation within synovial tissue

Pilot study to determine feasibility and validity of two methodologies

1. <u>The montage method:</u> As the montage method randomly samples hpfs within each synovial section a feasibility study was conducted to determine the optimum sampling intensity (via assessment of inter and intra sample variability) necessary to accurately characterise the inflammatory infiltrate. Thus in order to determine the minimum number of hpfs required to obtain an accurate mean of area fraction occupied by mononuclear cells in aggregate form within a tissue, two patients were identified one with a highly inflamed synovium and one with a less inflamed synovium. For each patient a section from each available site was digitised and all hpfs within each section sampled exhaustively.
2. <u>The overview method:</u> the same two patient samples identified above were also analysed with the overview method by a second observer blinded to initial results.

Results obtained from each methodology generated from the pilot study were then compared in order to determine consistency in methodology.

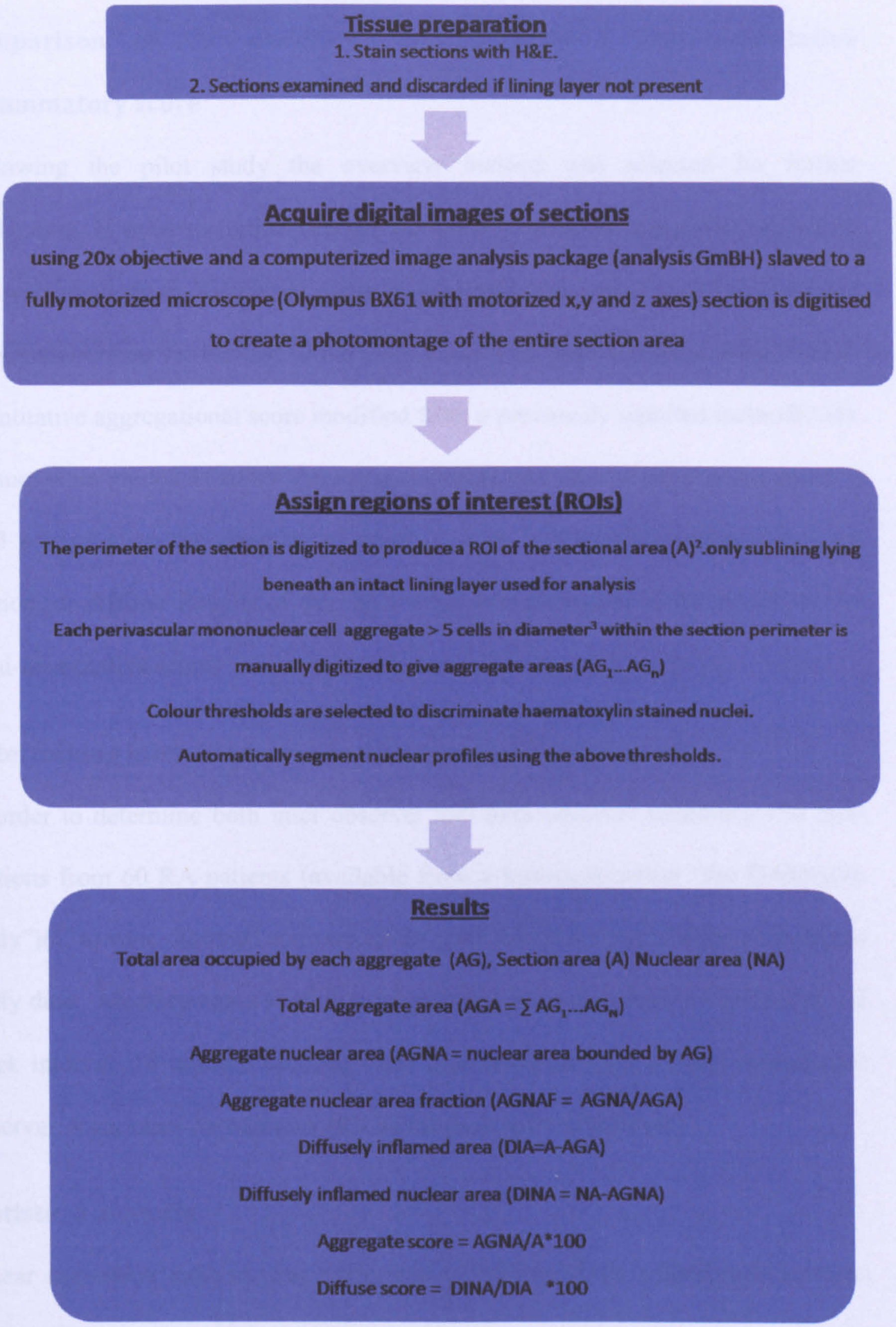


Figure 20: Flow chart illustrative of the overview method for quantifying degree of mononuclear cell aggregation and diffuse cellular infiltration within synovial tissue

Comparison of the overview method with a semi-quantitative inflammatory score

Following the pilot study the overview method was selected for further application. In order to further validate the QAS 25 sections of paraffin embedded synovial tissue from 7 different patients were assessed using the QAS. A second independent observer blinded to the QAS result then graded tissues using a semi-quantitative aggregational score modified from a previously reported method(334). Tissues were graded as either aggregate or diffuse and then as either aggregate 1, 2 or 3 according to the presence of grade 1, 2 or 3 aggregates within the tissue section, or diffuse. Results of the QAS were then compared to the results of the semi-quantitative score.

Determining inter and intra observer reliability for the QAS

In order to determine both inter observer and intra observer reliability 150 5μm sections from 60 RA patients (available from a historical cohort "the DAMAGE study")(176) were digitised and underwent QAS analysis by an observer blinded to study data. Measurements were then repeated by the same observer following a 2 week interval. 10 random sections were then analysed by a second independent observer. Intra-class correlations (ICCs) for each were determined.

Statistical analysis

Linear regression analysis was performed to determine the relationship between MC and DIA in determining nuclear number. Spearman's correlation coefficients were used to investigate the correlation between nuclear area/number (DIA) and nuclear number (MC). ICCs were used to analyse reproducibility of the QAS.

All results were analysed using GraphPad Prism version 3.03 for Windows, GraphPad Software, San Diego California USA. A p value <0.05 was considered statistically significant

Results

Digital image analysis reliably determines number of nuclei within synovial tissue sections

In order to determine whether DIA was as reliable technique as MC (the gold standard) in determining nuclei number 5 random areas within digitised sections of 12 synovial tissue samples were outlined as ROI with a digitising tablet (Figure 18). The number of haematoxylin stained nuclei within each ROI was counted manually and a second independent observer then used DIA to determine the number of nuclei within the same ROI. A close correlation between MC and DIA for determining nuclear number was observed (r^2 0.98, linear regression) validating the technique of DIA for determining nuclear number within our laboratory (Figure 21). A similar analysis was subsequently performed on the same tissues correlating number of nuclei within aggregate areas of tissue as determined by MC and DIA and the tight correlation between MC and DIA was not preserved ($r^2$0.51). This poorer correlation reflected the tightly packed nuclei without distinct borders within a number of densely packed aggregates, in which the Image analysis programme was unable to distinguish individual nuclei. Thus area occupied by nuclei within each aggregate was correlated with number of nuclei as determined by MC, and as a tighter correlation was seen (r^2 0.85). Therefore area occupied by nuclei rather than number of nuclei was chosen as the methodology of choice.

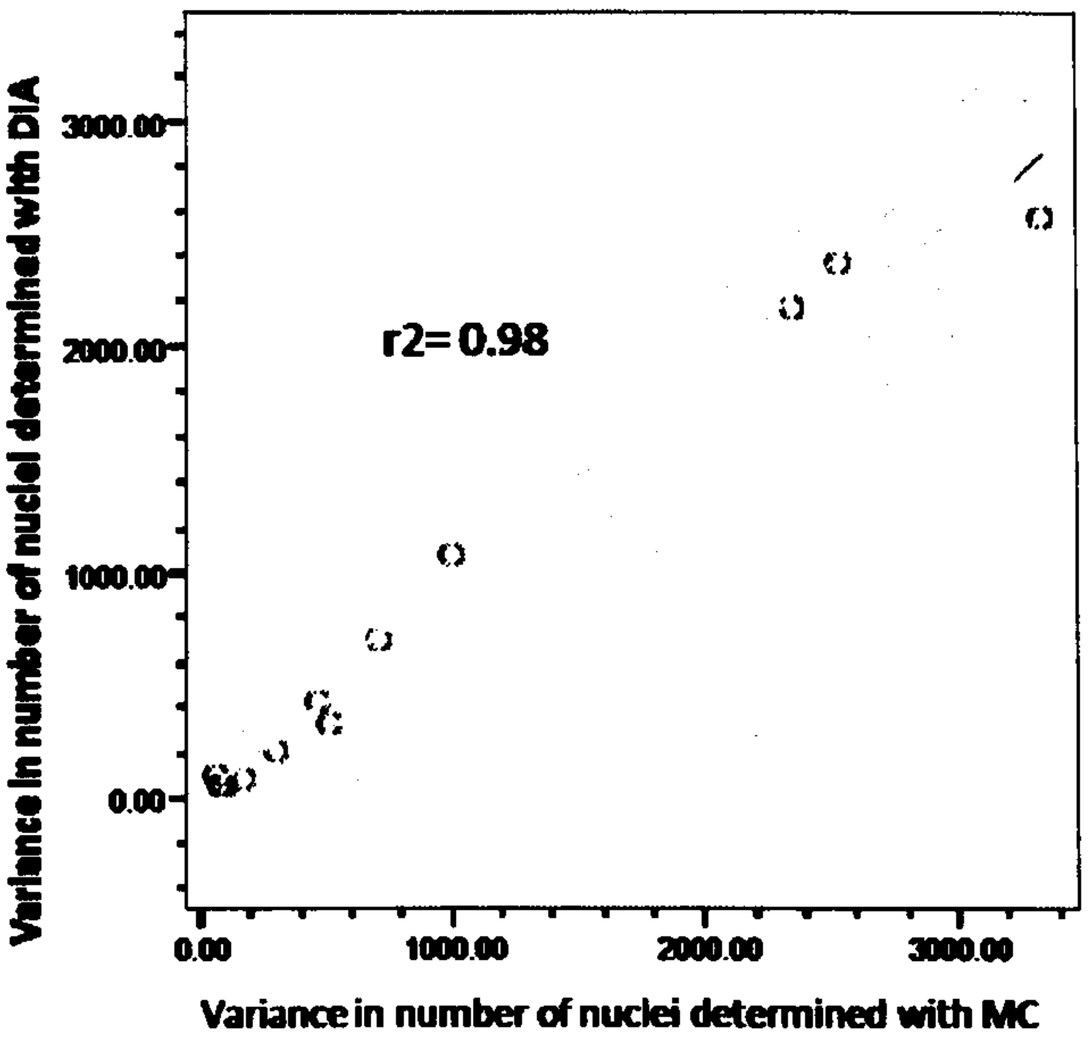


Figure 21: Linear regression analysis comparing variance in number of nuclei determined by DIA and number of nuclei determined by MC.

12 sections of synovial tissue were digitised and the number of nuclei within specific delineated regions of interest within the tissue determined firstly by manual counting (MC) and then using digital image analysis (DIA). A tight correlation was seen between MC and DIA demonstrating reliability for the technique. r^2 value shown. Lines represent 95 and 99% confidence intervals

The highest correlation between manual counting and digital image analysis is seen when synovial sections are stained with toludine blue

In order to quantify the effect of staining technique on reliability of measurement, number of nuclei as determined by MC was compared to area fraction of nuclei determined by DIA within aggregate areas within sections stained with H&E, Toludine Blue and the B cell marker CD20 (developed with vector red). Three aggregates within each section were outlined as regions of interest within each of 3 sequentially cut 5μm synovial sections from 3 different patients and nuclei quantified for each different staining method. The highest correlation between MC and DIA was seen when sections were stained with toludine blue (r 0.99). A relatively poor correlation was seen when sections stained with CD20 were examined (r 0.80) and a good correlation when H&E stained sections were examined (r 0.98) (Table 7).

Table 7: Spearman's correlation coefficients (and p values) for correlations of Digital Image analysis vs manual counting using different histological stains.

In order to determine whether different staining techniques affected reliability of DIA to quantify nuclei, three sequentially cut sections of paraffin embedded synovial tissue from three different patients were stained with haematoxylin and eosin (H&E), toludine blue and the B cell marker CD20. Manual counting (MC) was then used to determine number of nuclei and digital image analysis (DIA) to determine area occupied by nuclei within each of three aggregates defined within each tissue section. Results show correlation between number of nuclei counted using MC and nuclear area defined by DIA. Results were analysed using Spearman's rank, p values <0.05 were taken as significant.

	DIA vs MC
H&E	0.98 ($p<0.001$)
Toludine Blue	0.99 ($p<0.001$)
CD20	0.80 ($p<0.05$)

The overview and montage methods demonstrate internal consistency in determining area fraction of tissue occupied by mononuclear cells in aggregate form but the montage method is highly time intensive

The montage method:. In order to determine the minimum number of randomly chosen hpfs required to represent accurately the overall aggregational tendency of the tissue 13 synovial samples were analysed exhaustively (Table 8) and the results expressed as a plot of the progressive sample mean area fraction vs. number of hpfs examined (Figure 22). The number of hpfs at which the sample mean lies consistently within the 95% confidence interval for the sample population was determined and used as the basis for the number of fields required for examination in subsequent studies. Increasing the sample number further would produce no significant improvements in the accuracy of the mean and would increase the costs of analysis disproportionately.

For tissue (a) the optimum number of hpfs examined was 35 and the area fraction of tissue occupied by mononuclear cell aggregates is 0.03 (Figure 22a). For tissue (b) the optimum number of hpfs was 46 and the area fraction of lymphocytic aggregates 1.79 (Figure 22b). Thus a minimum of 46 hpfs are required for accurate determination of lymphocytic aggregation within each sample.

Overview method: the same patient samples (Table 8) used for the montage method were also analysed with the overview method (Table 9) by a second observer blinded to initial results.

The pilot study demonstrated that use of the montage method would be unfeasible for practical application to clinical studies, as the analysis of 46 hpfs would be

highly time intensive. When results for mean area fractions of mononuclear cells in aggregate form obtained by each method were compared however no significant difference was seen between the methodologies thus demonstrating internal consistency. On this basis the overview method was chosen for all subsequent analyses and is referred to from herein as the quantitative aggregational score (QAS).

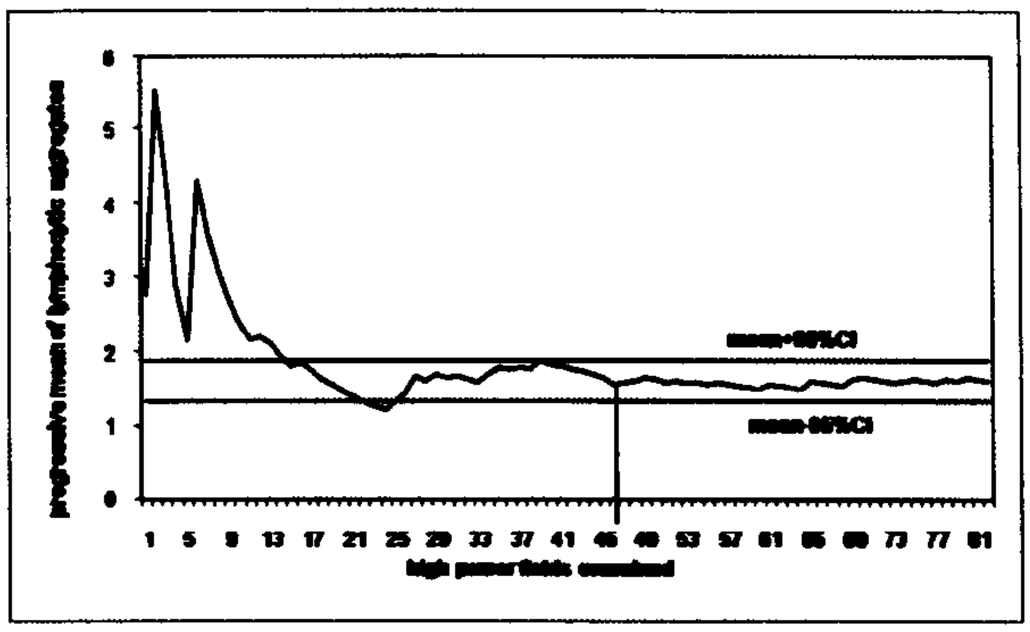
Table 8: Number and sites of synovial samples analysed for pilot study

2 patients were identified for inclusion in the pilot study to compare use of the overview and montage methods for quantifying cellular organisation within synovial tissue.

	Biopsy site	No of biopsies per site
Patient 1 (inflamed tissue)	SPP	2
	CPJ	2
	MPM	2
Patient 2 (less inflamed tissue)	SPP	2
	SPP	2
	CPJ	2
	MPM	1

SPP suprapatellar, CPJ cartilage pannus junction, MPM medial perimeniscal region

a.



b.

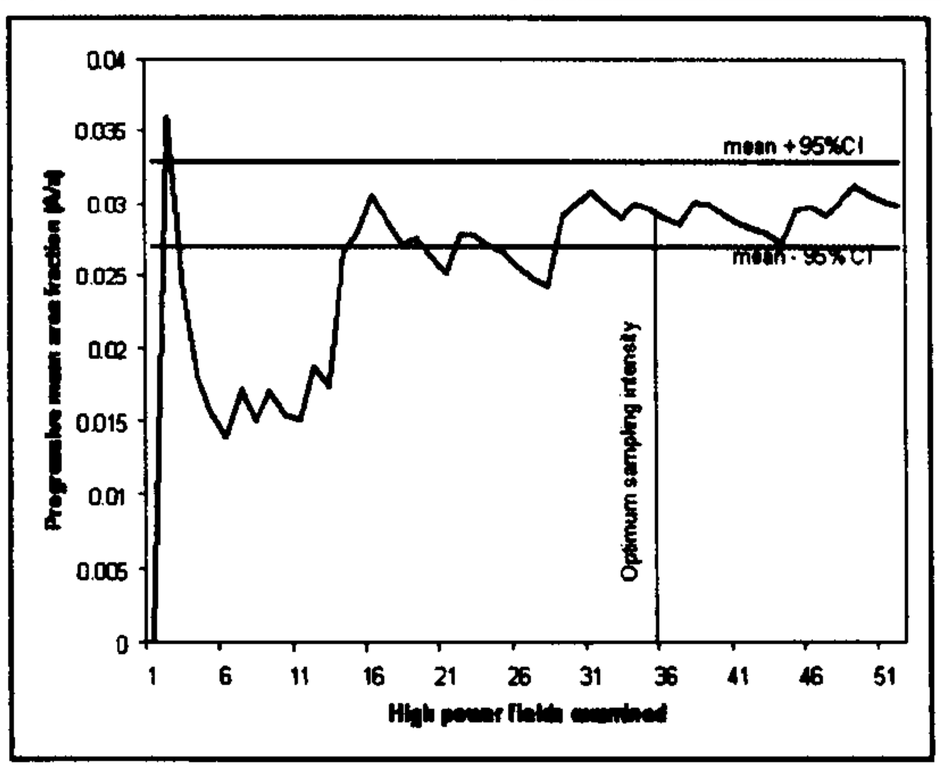


Figure 22: Progressive mean plot of area fraction of mononuclear cell aggregates within a sample of RA synovium versus number of high power fields examined.

The progressive mean is an empirical tool used to illustrate intra-sample variability. As the number of hpfs (x-axis) increase the rate of change of the mean reduces to a point where further sampling provides no further increase in the accuracy of the parameter estimate (in example a. the optimum sampling intensity lies in the region of 35 fields (vertical drop line, in example b the same is true at 46 fields). (Blue horizontal lines show mean +/- 95% confidence for the population mean) Mean for tissue a is 0.03 (expressed as area fraction) and 1.65 for tissue b (expressed as % of total area).

Patient identification no.	Mean area fractions (+/- SD) of synovial tissue occupied by mononuclear cells in aggregate form		
	overview method	montage method	P value
1 (n=6)	2.88 (1)	3 (1.3)	>0.5
2 (n=7)	1.65 (0.7)	1.79 (0.8)	>0.5

Table 9: Results from the pilot study demonstrate internal consistency in both the overview and montage methods of analysis

Mean area fractions (+/- SD) of synovial tissue occupied by mononuclear cells in aggregate form were determined for 2 different patients. There is no significant difference between mean scores as determined by either the montage or overview method. N=number of samples examined per patient

A higher quantitative aggregational score correlates with a higher semi-quantitative aggregational score

In order to determine whether the QAS as determined by the overview method could reliably distinguish aggregate from diffuse tissues, and whether tissues with more organised aggregates generated higher aggregate scores, results from the aggregate score component of the QAS were compared to a previously published semi quantitative aggregational score(334). 25 sections from 7 patients were analysed in total and the results demonstrated that tissues classified as aggregate achieved a much higher grade in the QAS (Table 10), with the highest aggregates scores in tissues containing predominantly grade 3 aggregates, whilst tissues graded as diffuse had much lower aggregate scores.

Table 10: A higher quantitative aggregational score correlates with a higher semi-quantitative aggregational score

7 different patients were identified with widely varying inflammatory infiltrates. Synovial tissue from a number of sections from each patient were graded as either diffuse, aggregate 1, 2 or 3 according to a previously published semi-quantitative aggregational score (334). A second observer blinded to these results then assessed the tissues using the quantitative aggregational score (QAS) described within this chapter. Patients graded as aggregate had consistently higher aggregate score (AS) component of the QAS than those graded as diffuse.

Tissue (no sections sampled)	Mean AS per tissue (+/- SD)	Grade of tissue
19816 (5)	2.88 (1.41)	Aggregate 3
11958(3)	1.64 (1.51)	Aggregate 2
2735(2)	0.65 (0.54)	Aggregate 1
12449 (3)	0.08 (0.01)	Diffuse
2450 (2)	0	Diffuse
9414 (7)	1.65 (1.72)	Aggregate 2
5589 (3)	0	Diffuse

The QAS is a highly reproducible pathological scoring system

In order to determine whether both components of the QAS, the aggregate (AS) and diffuse scores (DS), were reproducible, intraclass correlation coefficients (ICC) were determined for both intraobserver (n=152) and interobserver variability (n=10). Excellent reliability in measurement was demonstrated with ICCs >0.9 for both scores and both measurements (Table 11), suggesting that the QAS would be a sensitive measure of variability within the synovial membrane.

Table 11: Intraclass correlation coefficients (ICC) for inter and intra observer variability for aggregational score and diffuse score

In order to determine interobserver variability 150 sections of synovial tissue underwent scoring using the QAS, generating both aggregate (AS) and diffuse scores (DS). Scoring was repeated by the same observer following a two week interval. In addition in order to determine intra observer variability a second independent observer analysed 10 randomly chosen sections.

	AS ICC	DS ICC
Inter observer variability (n=150)	0.93	0.97
Intra observer variability (n=10)	0.92	0.90

Discussion

Predicting the prognosis of individual RA patients is at present difficult and new prognostic markers are urgently needed in order to tailor the most intensive expensive treatments to those most in need. The synovial membrane, as the primary site of inflammation in RA, shows potential as a biomarker and as such a number of synovial histological scoring systems have been developed although none currently has been shown to predict reliably prognosis(455). A number of factors, including the use of less sensitive semi quantitative scoring methods may have affected these results, as well as the inclusion of a number of non inflammatory measures within the scores. Aggregate structures within the synovial membrane have been associated with a poorer prognosis in cross sectional studies and as such their identification is a putative biomarker(421), with an emphasis on quantification as larger aggregates have been associated with functionality(334). DIA is the preferred means of analysis of synovial tissue for clinical trials, as it is an efficient method of analysing large quantities of tissue and further has been demonstrated to be sensitive to pathological change within the synovial membrane (444;445;674;677). Thus the aim of this study was to develop and validate a quantitative aggregational scoring system using DIA capable of accurately and sensitively determining organisational tendency of the inflammatory cells infiltrate within rheumatoid synovial tissue for use in clinical studies.

I first validated the use of DIA to determine number of nuclei within our laboratory, and as has been shown by other groups(444), demonstrate that DIA approaches the reliability of the gold standard MC in determining nuclear number on H&E stained tissue. It is important to note that if densely infiltrated aggregates were analysed where nuclear borders overlapped, it became difficult for the image

analysis programme to distinguish separate nuclei. A more representative measure of cellular infiltration was area fraction of tissue occupied by mononuclear cells and this was subsequently used. As nuclei are discriminated using a colour threshold technique I also investigated the effect of different staining techniques on DIA reliability in detecting nuclear number. I found that staining synovium with toludine blue produced equivalent results to those obtained with H&E, but a poorer correlation was noted when counting numbers of CD20+ B cells identified with IHC and developed with vector red. This variation is likely the result of a less distinct histogram produced when discriminating nuclei within tissue following IHC staining, a result of a wider red spectrum. Thus my approach to developing a scoring system used H&E stained tissues, a preferable stain to toludine blue as the implementation of the scoring system would include historical cohorts of patient tissues already stained with H&E.

Two scoring systems were developed, the montage and the overview method. The initial step for both methods was the generation of a digitised image of a tissue section. The montage method randomly sampled a number of hpfs within the tissue and determined the area fraction of mononuclear cells within each hpf in aggregate form. As only a fraction of the total area of section was sampled it was thought that this would produce a highly efficient means of sampling large volumes of tissue, in fact the converse was true and as data produced from a pilot study demonstrated a minimum requirement of 46 hpfs to be examined in order to determine a mean for the whole tissue, this technique was determined unfeasible for future practical application. The overview method, paradoxically, although analysing the whole tissue section was significantly less time consuming. The pilot study also demonstrated internal consistency between both methodologies,

i.e. the same result was produced if the tissue was analysed by either method. Thus the overview method was adopted as my method of choice. Analysis of synovial tissue with the overview method resulted in two scores, the aggregate score (AS) and the diffuse score (DS), the AS reflecting the area fraction of whole tissue occupied by mononuclear cells in aggregate form and the DS reflecting the area fraction of tissue occupied by mononuclear cells in the remaining tissue.

The overview method, subsequently referred to as the quantitative aggregational score (QAS) was subsequently validated against a number of measures. By comparing the QAS with a semi-quantitative inflammatory score, I demonstrated that tissues with a high degree of inflammatory cell organisation (as determined by the semi quantitative score) had a higher AS. In addition I also noted that one patient received a classification of diffuse using the semi-quantitative aggregational score and conversely received a positive AS. This was a result of a much more thorough analysis of synovial tissue using the QAS and so the likely identification of more aggregates and further tissue being graded as aggregate in the semi-quantitative methodology only if aggregates were the predominant infiltrate, rather than an occasional grade 2 aggregate leading to a positive AS when using the QAS. It remains to be seen whether either the semi-quantitative histological score or the QAS holds potential as a prognostic marker, or indeed whether the QAS is a superior histological score, although a study investigating this is currently underway in early arthritis within our group. I also investigated inter and intra observer variability and demonstrated that the QAS had excellent reliability in measurement for both the DS and AS, suggesting that the QAS would be highly sensitive to change within the synovial membrane.

In summary I have developed a novel QAS for rheumatoid synovium that determines both the degree of aggregational tendency of infiltrating mononuclear cells within synovial tissue and the degree of diffuse cellular infiltration and have demonstrated this score to be highly reliable, suggesting that it would be highly sensitive to change within the synovial membrane and thus of practical application in clinical trials.

Chapter 4: A Novel Quantitative Histomorphometric Score for Rheumatoid Synovium: Clinical, Biochemical, Synovial and Imaging Correlates from the Damage Study Cohort

Introduction

The rheumatoid synovial membrane is now acknowledged to be critical in driving rheumatoid arthritis (RA) pathogenesis. This concept is further strengthened by the demonstration that synovial biomarkers do not alter in the absence of effective therapy, unlike clinical assessment using disease activity scores (DAS) which have been shown to fluctuate with placebo therapy(678). Further the development of minimally invasive synovial biopsy techniques(679) has made the acquisition of tissue, including sequential biopsies, for both clinical studies and randomized control trials (RCTs) feasible and highly desirable.

Until recently data correlating ectopic lymphoneogenesis (ELN) with clinical phenotype was limited to small patient cohorts with samples from arthroplastic joints and thus end stage disease, with no data available from early and established disease. Furthermore no reports have investigated the role of ELN as a prognostic marker for joint damage progression. In addition although the pathogenic potential of ELN is currently causing much debate in the literature (426) a crucial question in addressing pathogenicity remains unanswered i.e. whether the distribution of ELN varies significantly throughout the joint.

Critically, the debate surrounding the role of ELN in RA has taken on increased importance as a pivotal role for B cells in the pathogenesis of RA, including in the synovial compartment, has been highlighted by the efficacy of B cell depleting agents(467). Moreover recent evidence suggests that the presence of ELN defines patients resistant to anti-TNF therapy(387), and that B cell infiltrates within the synovial membrane may play an important role in determining response to B cell depletion therapy(424;552;680).

In order to reliably compare synovial biopsies, both sequentially and between patients, I developed and validated a pathological scoring system, the quantitative aggregational score (QAS), capable of quantifying both the degree of aggregation (aggregate score [AS]) and diffuse infiltrate (diffuse score [DS]) within synovial tissue (Chapter 3). As a number of crucial questions remain regarding both the role of ELN in defining clinical phenotype and prognosis I aimed to use both clinical data and synovial tissue from a historical cohort of patients (the DAMAGE study cohort (176)) to test the hypothesis; whether in established RA, the level of synovial lymphoid organization predicts clinical phenotype, synovial cellular and cytokine levels, and joint damage progression. Hence, allowing not only the identification of patients in poor prognostic groups but also the identification of patients requiring aggressive therapy. Further, as the DAMAGE study patient cohort had synovium obtained arthroscopically from three distinct sites within the knee I also aimed to test the hypothesis that synovial inflammation (as determined by AS and DS) did not vary significantly throughout the knee joint

Aims and Objectives

Using both clinical data and synovial tissue available from an historical cohort of patients (the DAMAGE study cohort) the aims of this study were to determine whether:

i. There was significant variation in AS and DS throughout the joint
ii. Lymphocytic organisation correlates with local synovial cellular and cytokine parameters
iii. Lymphocytic organisation predicts clinical phenotype and joint damage progression

Materials and methods

Patients

Tissue and clinical data were available from 52 patients within the DAMAGE study cohort, described in detail in (176). All patients fulfilled the 1987 revised ACR criteria for RA(12). Patients were all naive to biological therapy and took a range of disease modifying drugs. Oral corticosteroids and non steroidal anti-inflammatory drugs were allowed. All patients gave their informed consent for biopsy and the study received approval from the Ethics of Human Research Committee of St George Hospital, Sydney, Australia.

Clinical, radiological and laboratory evaluation

Disease characteristics assessed included disease duration and the presence of IgM RF as determined by latex agglutination and anti-cyclic citrullinated peptide (anti-CCP) antibodies as measured by the anti-CCP2 ELISA. Disease activity was measured by baseline measurements of the 28 swollen and tender joint count, DAS score, erythrocyte sedimentation rate (ESR), platelets and C-reactive protein levels (CRP). Joint damage progression at baseline and 24 months was assessed by comparison of radiographs of hands and wrists using a modified Larsen score as previously described(176) and magnetic resonance imaging (MRI) assessment of the dominant-hand metacarpophalangeal (MCP) joints.

Synovial biopsy samples

At baseline patients had undergone arthroscopy of the knee joint under local anaesthetic. Multiple synovial biopsy samples were obtained from predetermined sites (suprapatellar pouch (SPP), medial perimeniscal (MPM), and cartilage–pannus junction (CPJ)), as previously described (21).

Synovial biopsy samples were either immediately snap-frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction or placed in 4% formalin for histology. After paraffin embedding 5μm thick sections were cut and mounted onto glass slides.

For histological assessment, sections were initially given a simple classification as either aggregate or diffuse. Aggregate sections had at least one identifiable grade 2 (or 3) aggregate using a previously described scoring system according to radial cell count (334) and diffuse sections only grade 1 aggregates or no lymphocytic organisation. Next, using DIA, sections were digitized and scored using the overview method (Chapter 3) to determine both the DS and AS.

Variability of the score throughout the joint

To determine whether there was significant variation in AS and DS between biopsies taken within the same site and between biopsies taken at different sites within the knee joint both AS and DS were determined at each available site and compared using intra class correlation coefficients (ICCs).

Agreement between repeated measurements of the same synovial biopsy ($ICC_{Repeated\ Measurements}$, ICC_{RM}) was determined by calculating the ICC.

Agreement between first versus second biopsies within the same site ($ICC_{Within-Site}$, ICC_{WS}) (SPP, CPJ and MPM) was calculated by ICC. Any reduction below ICC_{RM} may be attributed to variation in pathology between biopsies (rather than to measurement error).

$$ICC_{WS} = \sigma^2_{measurements} / (\sigma^2_{measurements} + \sigma^2_{within\text{-}site} + \sigma^2_{measurement\ error})$$

Agreement between synovial biopsies taken at different Sites ($ICC_{Between\text{-}Sites}$ = ICC_{BS}) was calculated using ICC. Any reduction of the ICC_{BS} below the ICC_{RM} may be attributed to variation in pathology between sites.

$$ICC_{BS} = \sigma^2_{measurements} / (\sigma^2_{measurements} + \sigma^2_{between\text{-}site} + \sigma^2_{measurement\ error})$$

Correlation of lymphocytic organisation with synovial cellular and cytokine parameters

5μm thick paraffin embedded synovial sections were stained using immunohistochemistry to detect B cells (anti-CD20) and macrophages (anti-CD68). After Ag unmasking using Target retrieval solution (pH 6; DakoCytomation) and incubation with protein block solution (DakoCytomation) primary Ab (anti-CD20 or antiCD68[Table 5]) was incubated at an appropriate dilution for 1 h at room temperature and biotinylated rabbit anti-mouse Ig was used as a secondary Ab. Following a 1-h incubation and three washes, HRP or AP-streptavidin-biotin complex (DakoCytomation) was added to the section and incubated for 30 min. After further washes colour reaction was developed with diaminobenzidine (HRP,DAB; DakoCytomation) or Vector red (for AP; Vector Laboratories). Numbers of both macrophages and CD20+ B cells per hpf per each slide were determined using manual counting and a mean number of cells per hpf per section determined. Significant differences in numbers of macrophages and B cells in aggregate and diffuse tissues was then determined.

Synovial biopsy levels of mRNA for IL10, TNFα, IFNγ and IL1β had been determined previously and the technique is described in (176). Briefly total RNA was extracted from snap-frozen synovial biopsy samples in parallel, using the Chomczynski technique then quantified and stored at -80°C before reverse transcription–polymerase chain reaction (PCR) analysis. Quantification was performed using an external standard curve, in a modification of a previously

reported technique (421). Cytokine mRNA levels in tissues graded as both aggregate and diffuse were then analysed for significant differences.

Correlation of lymphocytic organisation with clinical parameters

In order to minimise sampling error introduced by variability in cellular infiltration throughout the joint only those patients with synovial tissue available from at least 3 separate sites (SPP, MPM or CPJ) were chosen for analysis. 2 sections at least 50μm apart from each block containing tissue samples from each site were assessed using DIA to obtain a QAS. Mean scores for each site and for each patient were then calculated and correlated with clinical parameters (including DAS, CRP, ESR, platelets, disease duration, autoantibody status and degree of erosive damage over time). In addition significant differences in clinical parameters were examined in patients classified as either aggregate or diffuse.

Statistical Analysis

ICCs were used to analyse reproducibility of the QAS, to determine agreement between first versus second biopsies within the same site and to determine agreement between synovial biopsies taken at different sites. Multiple ANOVA was used to determine whether systematic differences existed between first and second biopsies within each site and between biopsies taken at different sites.

Differences in quantitative variables were analysed by the Mann Whitney U test. Spearman's rank correlation was performed to correlate pathological scores with clinical parameters. All the statistical analyses were performed using GraphPad Prism version 3.03 for Windows, GraphPad Software, San Diego California USA. A p value <0.01 was considered statistically significant.

Results

Clinical assessments and drug history

60 patients were recruited to the study and clinical data were available for all patients. 52 patients had synovial tissue suitable for histological analysis and were therefore used for validation of the score and to correlate lymphocytic organisation with synovial cellular and cytokine parameters. 29 patients had synovial tissue available from a minimum of 3 sites within the knee joint and so were used to assess correlation with clinical parameters. Data on radiographic progression (as determined by MRI and Larsen scores) was available from 44 patients.

The main demographic and clinical characteristics of patients have been published previously(176) and therefore a limited summary is given in Table 13. Significant differences in age, sex, disease duration, autoantibody status and levels of CRP between each of the groups were examined and no significant differences found ($P>0.05$).

Table 12: Demographic details, autoantibody status and disease duration of the 60 patients recruited to the DAMAGE study.

A total of 60 patients were recruited to the study. Clinical data was available for all patients, 52 had synovial tissue suitable for histological analysis and were used for determining variability of lymphocytic organisation throughout the joint and for correlating lymphocytic organization with synovial cellular and cytokine parameters. 29 patients had synovial tissue available from 3 sites throughout the knee joint and were used to correlate lymphocytic organisation with clinical parameters.

Pt ID	Sex	Age (yrs)	D D (yrs)	AutoAb status		Synovial sample	
				RF	CCP	Suitable (n=52)	≥3 sites (n=29)
1	M	66	2.66	+	-	n/a	n/a
2	F	68	0.112	+	n/a	n/a	n/a
3	F	73	0.54	+	+	n/a	n/a
4	F	49	11.7	+	n/a	a	n/a
5	F	49	29.7	+	+	a	n/a
6	F	42	19.7	+	+	n/a	n/a
7	F	42	2.75	+	+	a	n/a
8	F	41	2.75	+	+	a	n/a
9	F	40	13.7	+	n/a	a	n/a
10	F	61	15.7	+	-	a	a
11	F	55	3.78	+	+	a	a
12	F	52	12.8	+	n/a	a	n/a
13	F	36	7.84	-	+	a	n/a
14	F	37	0.665	+	+	a	n/a
15	F	69	1.32	+	+	a	n/a
16	M	69	1.41	-	-	a	a
17	F	56	13	+	-	a	a
18	F	65	12.9	+	+	a	a
19	F	57	1.41	+	n/a	a	a
20	M	78	3.11	+	+	a	a
21	F	40	10	+	+	a	a
22	F	37	0.095	+	n/a	a	a

23	F	63	14.1	+	-	a	n/a
24	F	49	0.714	-	-	a	n/a
25	M	83	4.19	-	-	a	n/a
26	F	80	13.1	+	n/a	a	n/a
27	F	50	10.1	-	-	n/a	n/a
28	F	73	5.19	+	n/a	n/a	n/a
29	F	61	12.2	+	+	a	n/a
30	F	62	0.845	+	+	a	n/a
31	M	64	0.249	-	-	a	a
32	M	78	10.2	-	n/a	a	a
33	F	37	6.28	+	-	a	a
34	F	54	2.28	+	-	a	a
35	M	49	1.07	+	+	a	a
36	F	43	12.3	+	+	a	a
37	M	46	4.32	+	+	a	a
38	F	73	5.32	-	+	a	a
39	F	50	2.33	+	n/a	a	a
40	F	62	1.51	+	+	a	n/a
41	M	47	7.92	+	+	a	a
42	F	52	7.26	+	+	a	a
43	M	54	9.43	+	+	n/a	n/a
44	M	74	0.517	-	-	n/a	n/a
45	M	32	4.07	-	+	a	a
46	M	77	8.49	+	+	a	a
47	M	54	0.996	+	n/a	a	a
48	M	61	1.91	-	+	a	a
49	M	55	6.17	+	n/a	a	n/a
50	F	55	1.43	+	n/a	a	n/a
51	F	60	5.5	+	n/a	a	n/a
52	F	56	10.5	+	+	a	a
53	F	68	10.5	+	+	a	a
54	M	77	7.51	+	n/a	a	n/a
55	F	64	2.6	+	+	a	a
56	M	78	0.273	-	-	a	n/a
57	F	76	0.29	-	-	a	n/a
58	M	70	0.331	n/a	n/a	a	n/a
59	M	68	0.323	-	-	a	a
60	M	60	0.451	+	+	a	a

Pt patient, F Female, M male, RF rheumatoid factor, DD disease duration, a available, n/a not available

Table 13: Baseline patient demographics and clinical characteristics of DAMAGE study (176) patients.

Column A shows all patients recruited to the study (n=60). Column B details the 52 patients used to determine variability of histological score throughout the joint and correlation of lymphocytic organisation with synovial cellular and cytokine parameters. Column C details the 29 patients with synovial tissue available from 3 sites within the knee joint and used for correlating lymphocytic organisation with clinical parameters.

	A (n=60)	B (n=52)	C (n=29)
Disease duration range (median/IQR) (years)	0.1-29.7 (4.1/9)	0.1-15.7 (4.3/10)	0.1-29.7 (4.07/10)
Age, mean +/-SD (years)	58+/- 13.3	57 +/-13.3	59 +/-12.5
Female (%)	67	65	56
Clinical Characteristics			
RF +ve (no (%)	70	76	75
***anti-CCP+ve (U/L) % (no)**	68 (35)	68 (26/38)	70 (17/24)
CRP, mean +/- SD mg/dL	17.6 +/-21.8	17.1 (+/-44.8)	17.6 (+/-23.7)
Drug therapy			
Receiving DMARD therapy no (%)	40 (67)	n/a	n/a
Receiving corticosteroids, no (%)	30 (50)	n/a	n/a

data available from 51 patients. RF, rheumatoid factor, CRP C reactive protein,

DMARD disease modifying therapy

Increased lymphocytic organization is associated with other measures of synovial inflammation

Individual components of the QAS, the AS and diffuse scores DS were compared to assess the relationship between degree of overall cellularity of tissue (as determined by the DS) and the degree of aggregation seen within a tissue. I found a close correlation (Spearman $r=0.69$, $p<0.05$) between increasing overall cellularity of the tissue (as determined by the DS) and increasing aggregation (as determined by the AS).

In order to investigate the relationship between lymphocytic organization and B cell and macrophage infiltration I compared cellular infiltration with B cells and macrophages in those tissues categorized as aggregate versus diffuse. I found a significantly higher infiltrate of both B cells (7.22 vs 2.46, $p<0.001$) and macrophages (7.22 vs 1.32, $p<0.0001$) in tissues classified as aggregate (Table 14), indicating that the presence of ELN is associated with a more inflammatory cellular infiltrate, a finding reported by other studies(388).

In addition I investigated whether the presence of ELN was associated with the differential expression of a number of key cytokines involved in disease pathogenesis, as has been suggested by previous data (421). I found no significant differences in the levels of IL10, IL17 or RANKL, and although levels of TNFα were higher in those tissues graded aggregate, significant differences were only detected for IL1β and IFNγ (Table 14).

Table 14: Relationship between cellular organization and measures of local synovial inflammation.

Tissues were classified as either aggregate or diffuse. Significant differences in gene expression levels of a number of key cytokines (as determined by RT-PCR) and number of macrophages (CD68) and B cells (CD20) in each group were determined.

	Aggregate	Diffuse	P value
Cytokines (fg/0.13µg total mRNA)			
Il-1β	253 +/-112	144 +/-101	**<0.01**
TNF-α	1359 +/-912	1022 +/-605	0.25
IL-10	1471 +/-1132	1460 +/-1027	0.9
IFNγ	24 +/-24	16 +/-23	**<0.05**
IL17	74+/-142	34+/-60	0.305
RANK Ligand	234+/- 218	234+/-205	1
Cellular markers (per mm^2)			
CD68	7.22 +/- 4.2	1.32 +/- 3.24	**<0.0001**
CD20	7.22 +/-6.4	2.46 +/- 3.3	**<0.001**

Values represent mean +/- SD, results were analysed using the Mann Whitney U test, p values <0.05 were taken as significant and are shown in bold face.

The aggregational and diffuse scores vary significantly at different biopsy sites throughout the joint

52% (15/29) of patients were identified as showing features of an aggregate pattern (defined as presence of at least one grade 2 or grade 3 aggregate). The mean AS was 0.216 (+/-SD 0.45) and the mean DS 3.52 (+/-SD 3). Grade 3 aggregates were only found in patients with mean aggregational scores greater than 1 (27%, 8/29 patients).

ICCs were used to analyse reproducibility of the QAS, to determine agreement between first versus second biopsies within the same site and to determine agreement between synovial biopsies taken at different sites.

For AS intra-observer reliability was excellent (ICC_{RM} 0.92) as reported in Chapter 3. Agreement between biopsies within each site (ICC_{WS} 0.46) and between different sites (ICC_{BS} 0.59) was only moderate suggesting that there was considerable variation between biopsies within and between sites in the knee joint (Table 15).

For DS intra-observer reliability ICC_{RM} was again excellent at 0.97, as reported in Chapter 3. Agreement between biopsies within the same site (ICC_{WS} 0.83) and between different sites (ICC_{BS} 0.79) were lower suggesting that there was some variation between biopsies within and between sites in the knee joint although this tendency was not as strong as for the AS (Table 15).

MANOVA was used to determine whether systematic differences existed between first and second biopsies within each site and between biopsies taken at different sites. MANOVA found significant differences in AS between biopsies taken at different sites (F 4.05, p 0.018) but not between biopsies taken at the same site or between assessments of the same synovial biopsy (Table 15). Figure 23 clearly

shows that AS were significantly higher at the MPM. MANOVA found no significant differences in DS within sites. However, variation between biopsies taken at different sites approached significance (F 2.93, p 0.055). Figure 23 indicates that Diffuse Scores were higher at the MPM, although not significantly so.

	Model		
	ICC	**Multiple ANOVA**	
Diffuse score		F	P
Repeated measurements (same biopsy)	0.97	0.40	0.848
Within site	0.83	0.58	0.446
Between site	0.79	**2.93**	**0.055**
Aggregate score			
Repeated measurements (same biopsy)	0.92	0.51	0.48
Within site	0.46	0.41	0.52
Between site	0.59	**4.05**	**0.018**

Table 15: Variability of aggregate and diffuse scores throughout the knee joint.

288 synovial tissue sections from the DAMAGE study patient cohort underwent digital image analysis and the degree of aggregation (AS) and diffuse infiltrate (DS) quantified. Synovial tissue sections came from multiple biopsy sites within the joint (suprapatellar pouch, medial perimeniscal and cartilage pannus junction) and from multiple biopsies within each of these sites. In order to determine whether there was significant variability in both AS and DS throughout the knee joint intraclass correlation coefficients (ICC) generated from repeated measurements of the same biopsy (determining reliability of measurement) were compared to ICCs between biopsies within each site and between sites. Repeated measurement of the same biopsy demonstrated excellent intra-observer reliability (high ICC), whereas ICC were lower for both within site and between site measurements for both AS and DS, suggesting considerable variation between biopsies both within and between sites. Multiple ANOVA was used to determine significant differences in AS and DS within and between sites and demonstrated significant differences in AS and DS between sites.

Significant p values are shown in bold

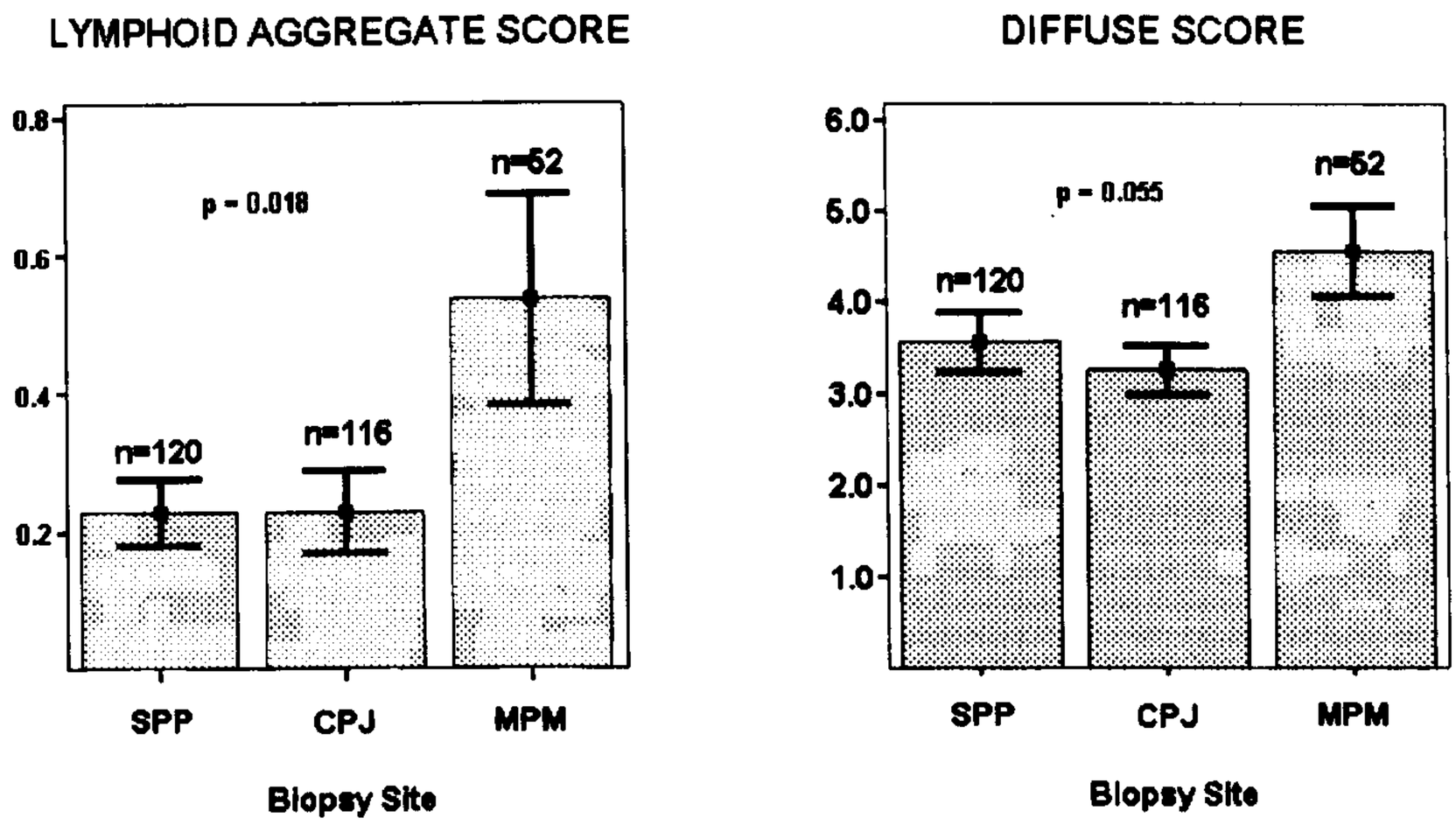


Figure 23: Aggregate and Diffuse Scores at Different Sites within the Knee joint

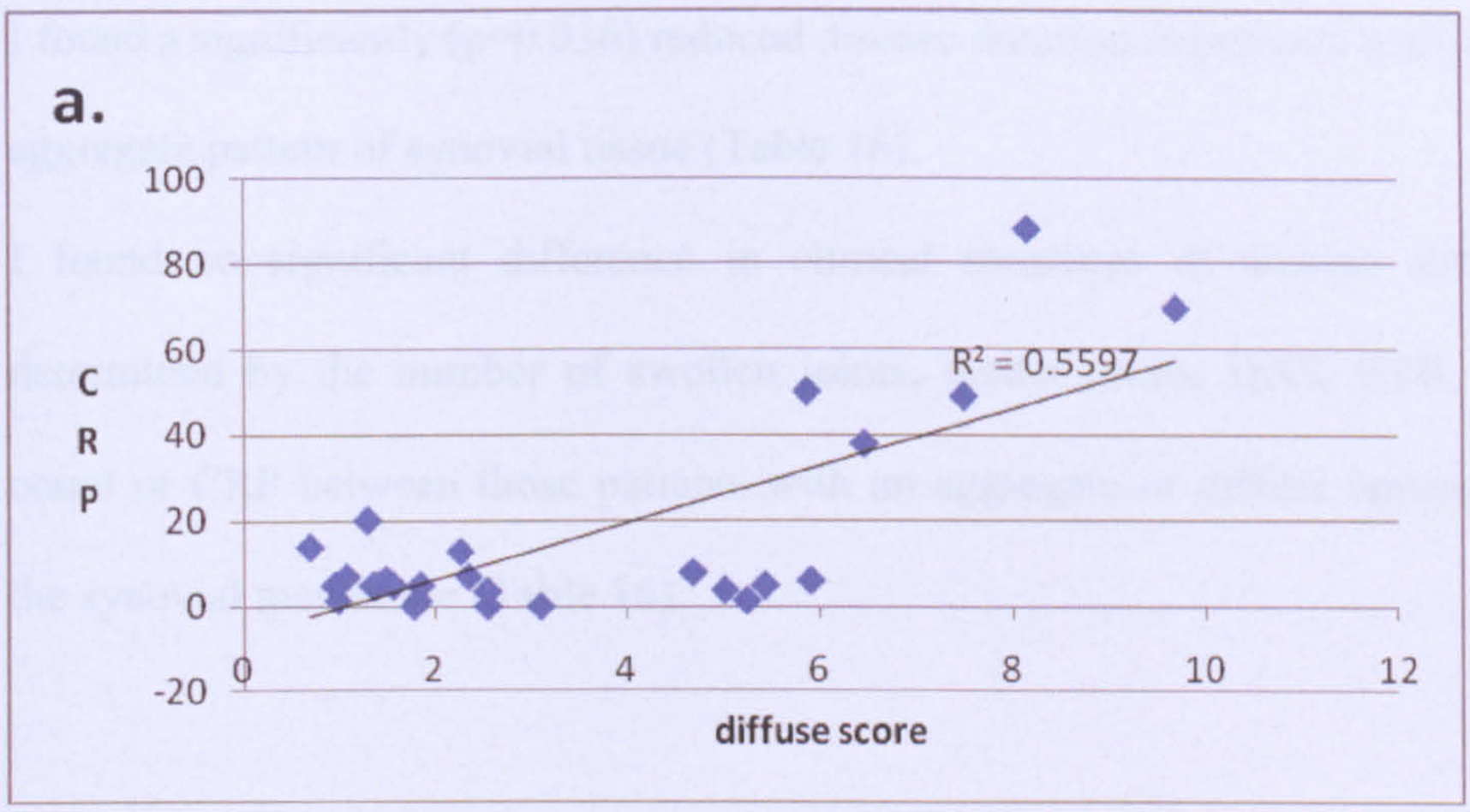
In order to determine whether systematic differences existed in both aggregate and diffuse scores from repeated biopsies from different sites within the knee joint (SPP: supra patellar pouch, CPJ: cartilage pannus junction, MPM medial perimeniscal) multiple (M)ANOVA was performed. The results clearly demonstrate a significantly higher aggregate score at the MPM, with a tendency to higher diffuse score at this site.

Bars show mean scores, error bars show standard error of the mean. P values are for MANOVA.

An increased aggregational and diffuse score correlates with elevated serum CRP, but not with the presence of autoantibodies in the serum of patients

I next investigated whether the degree of cellular organization within synovial tissue associated with a number of clinical parameters, for this component of the study 29 patients were included who had tissue data available from at least 3 sites throughout the knee joint. I found a significant correlation with serum levels of CRP and both increasing cellularity of tissue (diffuse score spearman r= 0.57 ($P<0.01$)) and increasing organization (aggregational score spearman r=0.51 ($P<0.01$) (Figure 24). There was no significant correlation found with either AS or DS and DAS, Larsen score, disease duration or swollen joint count.

There were no significant difference in serum levels of autoantibodies (either RF or anti-CCP antibodies), in patients with either an aggregate of diffuse appearance of the synovial membrane, a finding in line with other recent reports(388;416) (Table 16).



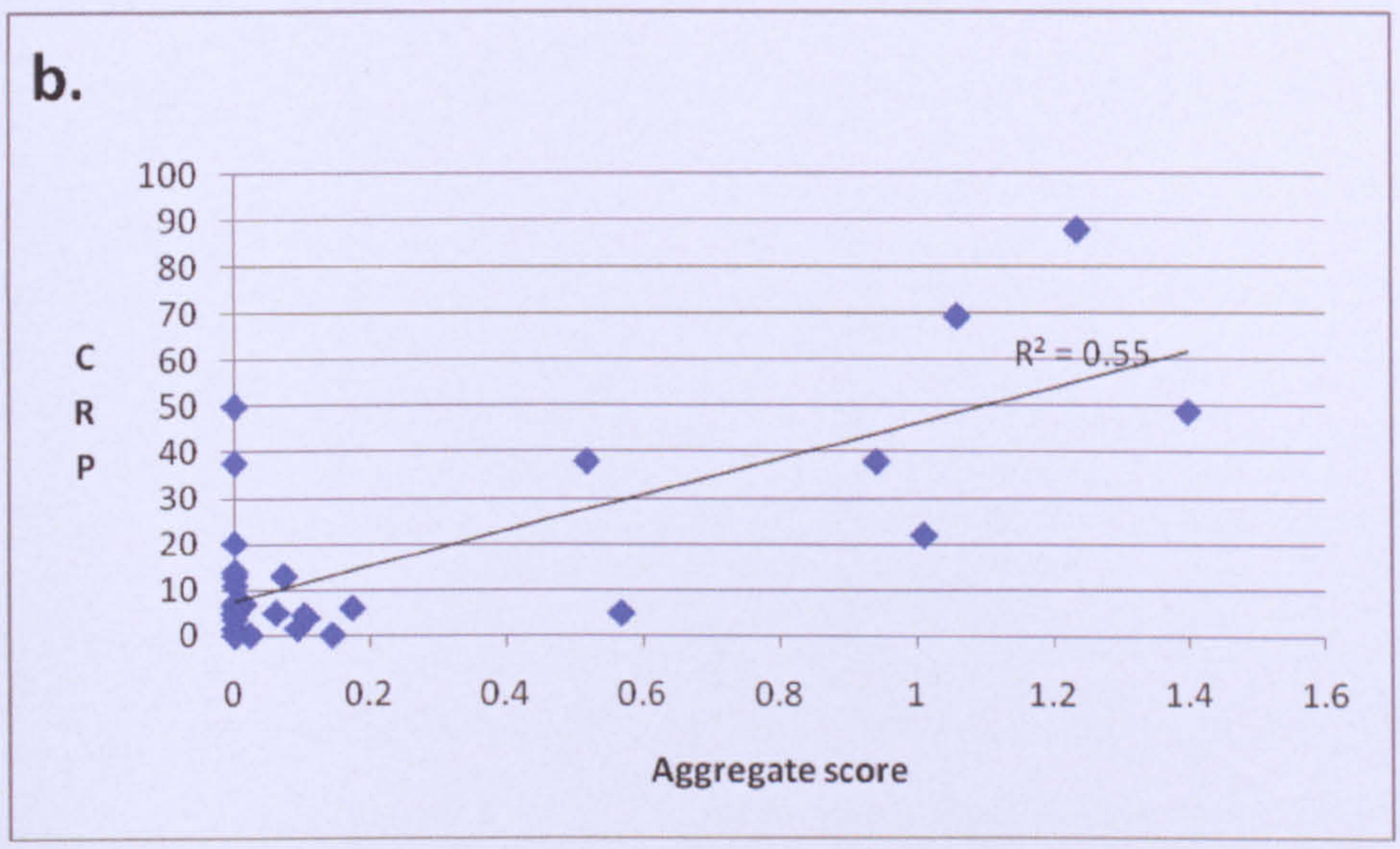


Figure 24. Correlation between aggregate and diffuse score and serum CRP

Aggregate and diffuse scores for each patient were generated and compared to serum CRP (C reactive protein). A significant correlation between both CRP and diffuse (a) and aggregate score (b) were seen ($p<0.01$). ($n=29$)

I found a significantly (p=0.036) reduced disease duration in patients with an aggregate pattern of synovial tissue (Table 16).

I found no significant difference in clinical measures of disease activity as determined by the number of swollen joints, tender joints, DAS, ESR, platelet count or CRP between those patients with an aggregate or diffuse appearance to the synovial membrane (Table 16).

Table 16: Association of lymphocytic organization and degree of cellular infiltration with baseline clinical parameters.

Patients from the DAMAGE study cohort (n=55) were classified as either aggregate or diffuse dependent on the presence of > grade 2 aggregates within synovial biopsies and significant differences in a number of baseline clinical parameters were investigated between the two groups.

	Aggregate	Diffuse	P value
RF	215 (+/-336)	280 (+/-468)	0.67
Anti-CCP antibodies (U/L)	153 (+/-158)	293 (+/-163)	0.46
Larsen score (0-150)	25.9 (+/-31.4)	24.1(+/-25.5)	0.76
Disease duration (years)	3.5 (+/-3.55)	7.7 (+/-5.274)	**<0.05**
Swollen joint count	7.9 (+/-5.71)	11.05 (+/-15.6)	0.98
Tender joint count	8.4 (+/-7.3)	11.3 (+/-8.7)	0.28
DAS	4.6 (+/-1.5)	5.4 (+/-0.8)	0.21
CRP (mg/L)	23.7 (+/-29.1)	9.70 (+/-9.73)	0.14
ESR	33.1 (+/-28.9)	36.6 (+/- 25.7)	0.76
Platelets	352 (+/-94.1)	315 (+/-143)	0.22

Values represent mean +/- SD results were analysed using the Mann Whitney U test, p values <0.05 were taken as significant and are shown in bold face. RF=rheumatoid factor, ACPA=anti-cyclic citrullinated peptide antibodies, CRP=C reactive protein, ESR=erythrocyte sedimentation rate, DAS=disease activity score

A higher aggregational score for synovial tissue does not predict the presence of erosions nor their development over a 2 year period

I next analysed whether baseline radiographic damage, at study entry, associated with the presence of aggregates within synovial tissue. I could find no significant differences in Larsen scores between those tissues classified as aggregate or diffuse (Table 16). Finally, as baseline radiographic scores do not incorporate erosive progression over time and as MRI has been demonstrated to be a more sensitive modality than conventional radiography to detect bone erosions (681), baseline aggregational and diffuse scores for patients determined to have progressed or not over a two year period as determined by MRI were compared. Although mean AS were higher in progressors vs non progressors (mean scores 0.3 *vs* 0.13), the result did not reach statistical significance. In addition the presence of ELN at baseline (as determined by the presence of at least one grade 2 or 3 aggregate) did not predict patients more likely to progress.

Discussion

The biological relevance of lymphocytic aggregates within the synovial membrane is currently attracting much debate in the literature(426), with recent evidence suggesting in established as opposed to end stage disease a lack of correlation with clinical phenotype(388). However there is increasing evidence to support a role for ELN in local autoantibody production(424) and for a pathogenic role for autoantibodies, in particular anti-CCP antibodies(481). This current interest in synovial ELN has been further stimulated by evidence demonstrating a role for such structures in predicting response to therapeutic intervention(387). Sensitive pathological scoring systems are therefore required in order to accurately determine both single and sequential assessments of lymphocytic aggregation within the synovial membrane. Semi-quantitative scores have been shown to be prone to bias and lack of sensitivity(674) and so their application to quantification of lymphocytic organisation within synovial tissue maybe limited. Overcoming such limitations may permit the use of lymphocytic aggregates as a useful prognostic biomarker for both disease progression and response to therapy.

I have developed a score for rheumatoid synovial tissue that quantifies, using DIA, both the aggregational tendency of tissue (AS) and overall degree of cellular infiltration (DS).

In this cohort of RA patients with established disease I identified 52% as positive for ELN, as defined by the presence of grade 2 aggregates or above, in the synovial membrane and identified 27% as positive for grade 3 aggregates. This result is significantly greater than recently reported(388) but is in line with other data in the literature(217).

My data demonstrates a direct correlation between an increased aggregational score and increased overall cellular infiltrate within synovial tissue. This concept of a central role for ELN in mediating synovial inflammation is further supported by a significantly increased number of both synovial macrophages and not surprisingly B cells in aggregate containing synovial tissue, a result in line with data from another group(388)and data demonstrating that following B cell depletion therapy a significant reduction is seen in not only synovial B cells but also T cells and macrophages(388). This suggests that the production of proinflammatory cytokines and chemokines by activated B cells, functioning in both attracting and retaining inflammatory cells, is critical in determining synovial inflammation.

Interpretation of the differential expression levels of a number of key inflammatory cytokines between tissues graded as aggregate and diffuse produced some interesting results. Firstly, I did not find significantly higher levels of TNFα in tissues graded as aggregate compared to tissues graded as diffuse as has previously been reported(421), although this was in end stage rather than established disease. This is interesting and requires corroboration by further studies, particularly as the presence of ELN (though not specifically higher levels of TNFα) has been demonstrated to predict resistance to anti-TNF therapy(387). Significantly higher levels of both IL-1β and IFNγ in aggregate tissues were found. IL-1β has already been demonstrated to predict joint damage progression and IFNγ conversely to confer protection from joint damage progression in this patient cohort(176). Both cytokines however have previously been associated with an aggregate synovial cell infiltrate(421). IL1β is a macrophage derived cytokine and its significant expression in aggregate tissues likely relates to the increased

association with increased macrophage numbers, demonstrated by the results of this and other studies(388). The differential expression of IFNγ, a T cell derived cytokine, within aggregate tissues maybe a result of T cells accumulating within GCs producing IFNγ, rather than traditionally IL4, as has been suggested previously(421). RANKLigand is known to be a critical cytokine mediating erosive damage in RA(682) and a potential pathway linking ELN to a more erosive disease pattern was the report demonstrating activated T cells expressing RANKLigand localized within sublining cell clusters(683). Although I found no differential expression between aggregate and diffuse tissues in the expression levels of RANKLigand, crucially, I were unable to report on expression levels of OPG, the decoy receptor for RANKLigand. As OPG is capable of blocking tissue destruction differential expression of this key cytokine between tissues would impact critically on the interpretation of these results with regard to a potential link between ELN and cartilage erosion. Unfortunately due to the historical nature of the DAMAGE study patient cohort no futher RT-PCR experiments were possible.

I demonstrate a significant correlation between increased aggregation (AS) and overall cellular infiltrate (DS) within synovial tissue and systemic inflammation (CRP) (388). Although a higher level of CRP was detected in patients with an aggregate vs diffuse appearance to the synovial membrane, this was not a significant result as has previously been reported(388) and likely reflects the smaller patient numbers in this cohort. The lack of correlation with tender and swollen joints is not surprising as clinical detection of synovitis has been demonstrated to be both unreliable and insensitive(684). A more robust measure maybe assessment of synovitis by either ultrasound using ultrasonography or MRI(685). Indeed, a study investigating a correlation between synovial

histopathological subtypes and ultrasound appearance of synovitis is currently underway within our group.

I found an association between a less organized inflammatory infiltrate and an increased disease duration. This supports the concept that ELN is not just limited to chronic end stage disease but, as has been, reported can be documented in early arthritis(449). I suggest that this relationship needs to be investigated in a cohort of patients specifically with early arthritis.

I do not report an association between the presence of ELN and the detection of autoantibodies in the serum of patients, in agreement with recent reports in established(416) as oppose to end stage disease(421). However, I do not believe, as has been suggested by others(388;416;426), that this demonstrates a lack of role for ELN in autoantibody production. Although I minimized sampling error examining tissue from multiple sites within a joint, the process of ELN is likely a dynamic modality between and within joints and thus limited bioptic sampling is unlikely to ever detect the true functionality of tissue. Importantly previous reports have suggested that different sites throughout the knee reflect different levels of inflammation (365;686), although these data has not been supported by a number of other studies(687;688) it is a particularly important consideration with the advent of minimally invasive synovial biopsying techniques, (679;689) which for the knee joint in general only sample the SPP. Indeed I demonstrate significant variability between biopsy sites (although not within sites) for both AS and DS, suggesting that limited sampling of one site from the knee joint may not be representative of the whole joint. In this study significantly higher levels of aggregation were demonstrated at the MPM rather than the SPP or CPJ. Further a direct comparison of sera (or indeed synovial fluid) with ELN within the synovial

membrane is compounded by the demonstrated production of antibodies at other ectopic sites such as the lung(386). Indeed when synovial tissue is examined directly, evidence points to a role for ELN in local autoantibody production(424). Thus there is a pressing requirement for further arthroscopic studies (able to access multiple sites within the knee) powered to determine the optimum site for acquisition and minimum amount of synovial tissue required to reliably classify a patient as of an aggregate or diffuse synovial pathotype.

As far as I am aware this is the first study to investigate the prognostic value of ELN in predicting radiographic progression in established RA. The prospective study of radiographic progression is of more biological relevance than quantifying radiographic damage at one time point, which is particularly difficult to interpret in patients of differing disease duration with exposure to multiple therapeutic agents. Further the use of MRI is demonstrably more sensitive than conventional radiography in detecting erosive disease(690). Despite this I was still unable to demonstrate a relationship between the presence of ELN and radiographic progression. I was hampered by the lack of progression in the majority of patients assessed (47%, 21/44), despite exposure to biological therapy. This lack of progression is likely a result of the inclusion of a variety of patients with widely varying disease severity on a diverse range of therapies. Further, as radiographic progression is a periodic event it would likely be more revealing to follow the patients over a longer time course. Further finally, as has already been discussed, significant variation in lymphocytic aggregation throughout the knee joint may have affected the result.

In conclusion, although the presence of ELN in this cohort of RA patients does not predict joint damage progression I suggest that the demonstrated association

with both synovial inflammation and increasing aggregation correlating with systemic inflammation (CRP) demonstrates a critical role for these structures in disease pathogenesis. I further suggest that this relationship needs to be investigated in patients with early arthritis, naive to all disease modifying drugs, a trial that is currently ongoing within our group, and that will use the QAS to assess the synovial membrane at baseline and sequentially.

Chapter 5: Ectopic Lymphoid Structures Support Ongoing Production of Class-Switched Autoantibodies in Rheumatoid Synovium.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory erosive polyarthritis with an associated significant morbidity and mortality (691). One of the hallmarks of the disease is the presence of circulating autoantibodies, such as rheumatoid factor (RF) (11) and anti-citrullinated protein/peptide antibodies (ACPA) (472-477), that have prompted the notion of an autoimmune pathogenesis. The presence of such antibodies, particularly anti-CCP antibodies, has been shown to be a poor prognostic factor linked with a higher erosive burden (529;530), while anti-CCP antibody titres have been reported to fall in line with clinical response to biological therapies (491). Additional support for a pathogenic role for anti-CCP antibodies comes from recent work in a mouse model of RA that indicated a direct role for anti-CCP antibodies in tissue destruction (481).

Physiologically, antigen-driven antibody responses take place within germinal centres (GCs) of secondary lymphoid organs where the processes of somatic hypermutation (SHM) and class switch recombination (CSR) of the Ig genes in GC-B cells occur, leading to affinity maturation and differentiation to memory B and plasma cells (692;693). However, recent evidence suggests that CSR and low-level SHM can also be sustained at extrafollicular sites in the spleen (202) and the gut (203). Both CSR and SHM are initiated by and critically dependent upon the expression of the enzyme activation-induced cytidine deaminase (AID) (197). Although the exact mechanism of action of AID is currently unclear (694), CSR occurs via excision of switch circles following the introduction of double strand breaks in the Ig switch regions of DNA, with the subsequent substitution of IgM

and IgD with IgG, IgE, or IgA (413), and hence production of antibodies with specific effector functions. AID also initiates SHM by introducing point mutations in the variable genes encoding the antigen-binding region of Igs (199), and affinity maturation then occurs through cycles of antigen-dependent selection in GC. Accordingly, AID expression in secondary lymphoid organs has been demonstrated to be restricted to GC-B cells actively undergoing CSR/SHM and to the recently described population of IF large(IF) large B cells (5;196;695). Therefore, delineation of its expression within tissue allows identification of whether and where B cells activate the molecular machinery responsible for the production of affinity-matured antibodies.

The presence of ectopic lymphoid structures resembling GCs and characterized by follicular dendritic cell (FDC) networks is a common finding in chronically inflamed tissues in several autoimmune diseases (383) including RA. GC-like structures have been reported to occur in the synovial membrane in approximately 25% of patients with RA (421). It has been shown that alongside the frequent recognition of disorganized cell clusters in the synovium, some of the sublining lymphoid aggregates can recapitulate qualitative features of secondary lymphoid organs, such as FDC networks, the in situ production of homeostatic chemokines, as well as the acquisition of variable degrees of T/B cell segregation or high endothelial venule development (217;334;404;416). However, whether ectopic lymphoid structures in RA can be functional and directly implicated in promoting the production of disease-specific and potentially pathogenic autoantibodies such as ACPA, or whether the synovium acts as a "reservoir" for long-lived plasma cells producing ACPA is at present unclear (416). Circumstantial evidence, such as the

presence of clonally mutated B cells from GC-like structures microdissected from RA tissues (415;423), suggests that an antigen-driven B cell response is taking place in the RA synovium. Likewise, the detection of ACPA in the synovial fluid of patients with RA (419;696), the demonstration that ACPA are concentrated at this site (420), and the detection of ACPA in the sera of SCID mice xenotransplanted with human synovium (418), support the possibility that ACPA might be directly generated in the synovium. These data, however, cannot rule out the possibility that already mutated, autoreactive B cells are preferentially recruited to the inflamed synovial tissue. In addition, lymphoid aggregates can also occur in the synovium of patients with other rheumatological conditions currently not associated with autoantibodies of known specificity(416), and further no correlation has been found between their presence and serum/synovial fluid ACPA(388;416). These concepts have been taken as an indication that these structures may not be functional and that B cell autoimmunity in RA synovitis may be independent of ectopic lymphoneogenesis (388;416;426).

Aims and objectives:

The purpose of this study was to investigate whether ectopic lymphoid structures in RA synovium are functional by determining whether they:

(i) Express AID, the enzyme required for SHM and CSR of Ig genes;

(ii) Support ongoing CSR and the production of high-affinity ACPA; and

(iii) Remain functional and promote B cell survival, proliferation, and autoantibody production in a RA/SCID chimera model devoid of any new

immune cell influx into the synovium and where the ongoing immunological activity of secondary lymphoid organs is excluded.

Materials and methods

Patients and samples

Following written informed consent, synovial tissue was collected from 55 patients with RA fulfilling the revised 1987 ACR criteria for RA (12). Tissues were collected consecutively within each group studied, which included arthroplastic joint surgery (40) and ultrasound-guided synovial biopsy (15). The majority of the patients were on methotrexate, and none were on anti-TNF therapy at the time of surgery. Demographic data are given for the study population in Table 17.

From 24 of the 55 patients who underwent joint replacement surgery between 1999 and 2005, only formaldehyde fixed, paraffin-embedded tissue was available and therefore was exclusively used for histological analysis. From an additional 25 patients (of whom ten underwent arthroplastic surgery and 15 underwent ultrasound-guided biopsy), enrolled between 2005 and 2008, paired paraffin and RNA samples were available and were used for histological analysis and QT-PCR. Finally, from six patients with RA undergoing joint replacement surgery, synovial tissue was obtained and stored in liquid nitrogen until use for transplantation into SCID mice.

Procedures were approved by the hospital Ethics Committee (REC/98/11/27 Guys and St. Thomas' NHS Trust and REC 05/Q0703/198 Barts and the London NHS Trust) and performed after written informed consent.

Table 17: Clinical and Histological Characteristics of RA Patients (*n* = 55)

Group	**Characteristic**	
	Age (y) mean ± SD	53 ±12.2 y
Clinical Characteristics	Female (%)	76.3%
	Disease duration (y) mean ± SD	13.2 ± 17.1 y
	Rheumatoid factor+ (%)	80%
Pathological scores of synovial tissues	Aggregate (CD21 network+) tissue (%)	29%
	Aggregate (CD21 network–) tissue (%)	31%
	Diffuse tissues (%)	40%
Patients with paired synovial tissue and serum n = **21**	ACPA+ (%)	71%
	Serum; ACPA+, tissue; AID/CD21L+ (%)	75%
	Serum; ACPA+, tissue; AID/CD21L– (%)	67%

Immunohistochemistry

A list of primary and secondary antibodies (Abs) used is reported in Table 5.

Histological Grading of Tissues

The histological grading of tissues and degree of lymphoid organization was assessed by immunohistochemical staining of sequentially cut sections of RA synovial tissue, as previously reported (334). Briefly, paraffin-embedded 5 μm sections underwent routine staining with haematoxylin and eosin in order to define the predominant histological pattern of RA synovitis as either diffuse or aggregate (421). The number of lymphocytic aggregates was counted in each section and graded according to a modified, previously published grading system (334;391) with grade 1 (G1) aggregates displaying a radial cell number between 2 and 5 cells, grade 2 (G2) between 6 and 10 cells and grade 3 (G3) greater than 10 cells.

Staining for CD3 and CD20 (using antibody dilutions of 1:50 and 1:20 respectively), following antigen retrieval with Target retrieving solution (DAKO), was used to analyse T/B cell segregation as previously reported (697). In addition, following proteinase K digestion (DAKO), single staining with CD21 at a dilution of 1:20 was performed to identify FDC networks characterising GC-like structures (697).

Based on the results of the CD21 staining and the presence of G2/G3 aggregates, samples were qualitatively classified as either diffuse, aggregate/CD21−, or aggregate/CD21+ as previously described (217). In order to minimize bias due to

cutting level, all blocks of tissue were cut and stained at three cutting levels 50 µm apart.

Relationship between Histological Grade of Tissue, Degree of Inflammatory Infiltrate Organization, and AID Expression within Tissues

Staining for AID was performed, as previously reported (675), on sequential sections of RA synovial tissue in order to correlate AID expression with the histological grading and degree of lymphoid organization. Briefly, after Ag unmasking using Target retrieval solution (pH 6, DAKO) and incubation with 20% rabbit serum (DAKO), incubation with the EK2-5G9 primary Ab was carried out at a dilution of 1:20 for 1 h at room temperature. This step, in turn, was followed by a 1h incubation with the biotinylated rabbit anti-rat Ig secondary Ab. The HRP–streptavidin-biotin complex (DAKO) was then added to the section and incubated for 30 min. The colour reaction was developed with diaminobenzidine (DAKO).

Relationship between AID and CD21L Expression, as Determined by QT-PCR and Histological Characterization of Synovial Tissues

Synovial samples were obtained from a further 25 patients with RA. Each specimen was divided into 2 parts; one was formalin fixed and paraffin embedded for immunohistology and the second was stored in a 10:1 v:v of RNA-later (Ambion) at −80 °C for RNA extraction and QT-PCR analysis. Histological characterization of the RA tissue was carried out as described above. Total RNA was extracted from the remaining portion of synovial tissue, using the RNeasy Mini Kit (Qiagen), with on column DNase I digestion to avoid genomic DNA contamination. cDNA was generated from 1 µg of RNA using the Thermoscript

RT-PCR System for First-Strand cDNA Synthesis (Invitrogen). QT-PCR was performed to detect mRNA expression levels of AID and CD21L together with CXCL13, LTβ, BAFF and APRIL using specific primers and probes (Table 6). The RT-PCR was run in triplicate with an equal loading of 20 ng of cDNA/well. Results were analysed after 40 cycles of amplification using the ABI PRISM 7900HT Sequence Detection System Version 2.1. Relative quantification was measured using the Comparative C_T (Threshold Cycle) Method. cDNA from peripheral blood mononuclear cells sorted for CD3 by fluorescence activated cell sorting (FACS) were used as a negative control for AID and CD21L, whereas cDNA from lymph node was used as a positive control.

Detection and Characterization of ACPA-Producing Cells within Rheumatoid Synovial Tissue

Citrullinated Fb (CFb) was generated as previously described with minor modifications (386;483). Briefly, plasminogen-depleted human Fb (Calbiochem: EMD BioSciences) was incubated at 0.86 mg/ml with 10 U/ml of rabbit skeletal muscle peptidyl arginine deiminase (Sigma-Aldrich) in 0.1 M Tris-HCl (pH 7.4), 10 mM $CaCl_2$, and 5 mM DTT for 2 h at 50 °C. The enzyme was inactivated by adding 2% SDS and heating at 100 °C for 3 min and removed by serial spinning and washing with 0.01 M sterile bicarbonate buffer in a 100 kDa Amicon filter device (Millipore). CFb was then biotinylated using NHS-LC biotin (Pierce Biotechnology). In addition, an aliquot of unmodified Fb of the same concentration as the CFb , was biotinylated and used as a negative control for all staining experiments.

To confirm effective citrullination and the specificity of CFb for ACPA Abs, two aliquots of CFb and uncitrullinated Fb were first separated by SDS-PAGE on 7.5% polyacrylamide gels. Proteins were then electrotransferred onto Hybond-C extra reinforced nitrocellulose membranes (Amersham). Membrane strips were probed with human sera (1:100) pooled from three ACPA+ patients with RA and three ACPA– patients with RA. A goat anti-human IgG peroxidase (Sigma) was used for detection of the primary Ab. Peroxidase activity was visualized using ECL Western blotting reagents (Amersham) following the manufacturer's instructions.

To detect and characterise ACPA-producing cells in the RA synovium, sequentially cut 5µm paraffin and/or frozen sections of five RA synovial samples representative of different degrees of histological organization (three aggregate/CD21+, one aggregate/CD21–, one diffuse) were chosen for analysis. ACPA-producing cells and B cell follicles were identified using double immunofluorescence with biotinylated CFb followed by incubation with streptavidin ALEXA-555 and CD20 followed by rabbit anti-mouse ALEXA-488 with subsequent DAPI counterstaining (386). AID+ FDC networks were identified on sequential sections by staining for CD21 using conventional IHC as described above and immunofluorescent staining for AID using streptavidin-ALEXA-555 following incubation with biotinylated rabbit anti-rat secondary Ab. Plasma cells producing ACPA were identified by performing double immunofluorescence for CD138 followed by goat anti-mouse ALEXA-488 and biotinylated CFb as described above. All sections were visualised using an Olympus BX60 microscope and epifluorescence.

Tissue Transplantation

A total of 56 samples of human synovium from six patients with RA undergoing arthroplasty (classified prior to transplantation histologically as two diffuse, two aggregate/CD21−, and two aggregate/CD21+) were transplanted subcutaneously into Beige SCID-17 mice as previously described (637;645). In this model, full engraftment of human tissue is reached at 7 d, and significant levels of ACPA antibodies in mouse sera have been described from a similar time point and up to 84 d (418). Four weeks post-transplantation I determined: (i) the histomorphology of the transplanted synovium with regard to the presence of CD21+ aggregates; (ii) the mRNA expression levels of AID, CD21L, and genes involved in ectopic lymphoneogenesis; (iii) the presence of ongoing CSR by assaying for the presence of Iγ-Cμ circular transcripts (molecular by products of IgM to IgG class switching); and (iv) the serum levels of human ACPA in the mouse circulation.

Animals were humanely killed at 4 wk and the grafts divided into two parts; one part was paraffin embedded for later histological examination and one part was stored in 10:1 v:v of RNA-later (Ambion) at −80 °C for QT-PCR. Grafts were examined macroscopically and histologically to confirm viability. In addition, transplanted synovial tissues were characterised by staining for CD21 and AID to identify persisting AID+ FDC networks. Proliferating B cells were identified by performing double immunofluorescence for Ki67 followed by goat anti-mouse IgG_1 ALEXA-488 and CD20 followed by goat anti-mouse IgG_{2a} ALEXA-594.

Finally, mice underwent a terminal bleed, and serum was collected and stored at −20 °C for subsequent ACPA analysis.

Gene Expression Analysis by QT-PCR and Detection of Circular Transcripts in Transplanted RA Synovial Tissues

RNA was extracted from each transplant, and cDNA was generated as described above. QT-PCR was performed to measure mRNA expression levels of AID, CD21L, APRIL, BAFF, TNFα, LTβ, and CXCL13 (Table 6).

Detection of Iγ-Cμ circular transcripts was performed on the same cDNA. Circular transcripts were detected following 30 cycles of RT-PCR amplification using the reverse primer Cμ (5′-GTTGCCGTTGGGGTGCTGGAC-3′) together with the forward primer Iγ (5′-GGGCTTCCAAGCCAACAGGGCAGGACA-3′) recognizing both Iγ1 and Iγ2 (the RT-PCR product was 557 bp) as previously reported (698). Before each RT-PCR, cDNA were denatured for 5 min at 94 °C. The PCR conditions were as follows: denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C, and extension for 1 min at 72 °C.

Detection of Human IgG ACPA in Human and Mouse Sera

ACPA were detected in both mouse and human sera using a commercially available anti-cyclic-citrullinated antibody (anti-CCP2) ELISA kit (Axis Shield), following the manufacturer's instructions. A positive result was taken as > 5 U/L.

Statistical Analysis

Differences in quantitative variables were analysed by the Mann Whitney U test when comparing two groups, and by the Kruskal-Wallis with Dunn's post test

when comparing multiple groups. χ^2 test with Yates' correction when required or Fisher's exact test when appropriate were used to evaluate associations of qualitative variables in the different groups. Spearman's rank correlation was performed to correlate expression levels of AID and CD21L mRNA in RA synovial grafts. All the statistical analyses were performed using GraphPad Prism version 3.03 for Windows (GraphPad Software). A *p*-value of < 0.05 was considered statistically significant.

Results

In the Rheumatoid Synovial Membrane AID Is Expressed Only in Association with FDCs

In order to investigate whether AID was expressed within the rheumatoid synovial membrane and its potential association with specific patterns of rheumatoid synovitis, I first performed an IHC analysis on sequential sections of synovial tissue in 24 patients with RA. I analysed AID expression in relation to the degree of lymphoid organization of the inflammatory infiltrate, including T/B cell segregation and FDC network formation.

As shown in a representative example in Figure 25, AID expression in RA synovium was invariably associated with the presence of FDCs (Figure 25A–H). This was the case even in CD21+ G3 aggregates that lacked uniform T/B cell segregation and distinct dark –light zones within the B cell rich area (423) (Figure 25A–1D). The pattern of AID staining within compartmentalized CD21+ G3 aggregates (Figure 25E–1H) was highly reminiscent of the pattern seen in secondary lymphoid organs, such as lymph node, with strong AID expression within GC B cells (Figure 25I–L). CD21+ FDC networks were observed in seven out of 24 patients (29%), with 100% of G3 aggregates displaying CD21+ FDC networks also expressing AID. Conversely, AID and FDC networks were not detectable either in smaller G1 or G2 aggregates or in diffuse tissues.

Figure 25: AID Expression within the Rheumatoid Synovial Membrane Is Restricted to Lymphoid Aggregates with FDC Networks

A representative example of sequential paraffin-embedded sections of LN and RA synovial membrane stained for (brown, A, E, I), T cells (CD3, brown, B, F, J), B cells (CD20, red, C,G, K) and follicular DCs (CD21, red, D,H, L). The presence of FDC networks was invariably associated with the expression of AID even in FDC+ grade 3 aggregates (arrow, A), without morphologically detectable dark and light zones within the B cell rich area (A–D). In more organised grade 3 aggregates (E–H), expression was more prominent and closely resembled that seen in secondary lymphoid organs, such as the LN (I–L) (original magnification was 20×). Scale bars 200 μm.

A
RA synovium
E
RA synovium
I
Lymph node
anti-AID
B
F
J
anti-CD3
C
G
K
anti-CD20
D
H
L
anti-CD21

QT-PCR Evaluation Confirms the Exclusive Association between AID and FDCs

In order to confirm whether AID expression was also exclusively associated with FDC networks at the mRNA level, I analysed synovial samples from an additional cohort of 25 patients with RA in which paired RA specimens for IHC and QT-PCR were available. In this cohort, I investigated the expression of CD21L, a marker exclusively expressed by FDCs (699), and AID transcripts. Indeed, AID mRNA expression was detected in all 12 of the CD21L+ RA samples but in none of the 13 CD21L− RA samples analysed (Figure 26A, $p < 0.0002$). Amplicons of the expected length (99 bp for AID and CD21L, and 171bp for β-actin, Figure 26B) were seen in samples positive for AID and CD21L by QT-PCR (lane 2) and human lymph node (lane 5), but not in RA samples negative for AID and CD21L (lane 3) or in the negative control (lane 4, cDNA from CD3 sorted cells).

In the same cohort of 25 patients I compared the expression of AID and CD21L mRNA by QT-PCR with histological characterization of the same piece of synovial tissue. I identified by IHC an aggregate pattern AID+/CD21+ in seven patients (28%), aggregate pattern AID−/CD21− in seven (28%) patients, and diffuse infiltrate negative for both AID and CD21 in 11 patients (44%). On the other hand, when the same samples were analysed by QT-PCR, I demonstrated a higher prevalence of RA synovial tissues expressing AID and CD21L mRNA (12/25, 48%). Notably, the increased proportion of RA synovial tissues expressing AID and CD21L mRNA was exclusively characterised by an aggregate and not a diffuse pattern, confirming that lymphoid structures are required for the expression of both AID and CD21L mRNA in the RA synovial membrane.

Expression of AID Is Associated with the Up-regulation of CXCL13 and LTβ within the Rheumatoid Synovial Membrane

As synovial tissues containing sites of ectopic lymphoneogenesis have been demonstrated to be closely associated with increased expression levels of CXCL13 and LTβ mRNA (217), I then sought to determine whether the expression levels of these and other key factors involved in lymphoneogenesis were also significantly associated with the up-regulation of AID and CD21L mRNA. As shown in Figure 26, AID mRNA+ tissues displayed significantly higher levels of CXCL13 and LTβ mRNA (Figure 26C and D) but not of the B cell survival factor BAFF (Figure 26F). Conversely, although APRIL mRNA showed an increase in AID+ synovial tissue, these differences did not reach statistical significance (Figure 26E).

Figure 26: AID mRNA within Rheumatoid Synovial Tissue Is Expressed Exclusively in Association with CD21L-Isoform Transcripts

QT-PCR was used to measure levels of transcripts of AID and CD21L in synovial samples from 25 patients with RA. Results were normalized for the endogenous control (human β-actin) and expressed as relative quantification.

(A) The presence of AID is restricted to those tissues expressing CD21L.

(B) Representative examples of the PCR products were run in a 1.8% agarose gel to ensure the presence of a single specific amplification product and confirm specficity of QT-PCR. Amplicons of the expected length (99 bp for AID and CD21L and 171 bp for β-actin) were observed in samples that gave positive signals at QT-PCR (lane 2), while no bands were detected in samples negative for AID/CD21L by QT-PCR (lane 3). No AID/CD21L amplification was detectable in negative controls (CD3-sorted cells, lane 4), while AID and CD21L were both detectable in control human lymph node (lane 5).

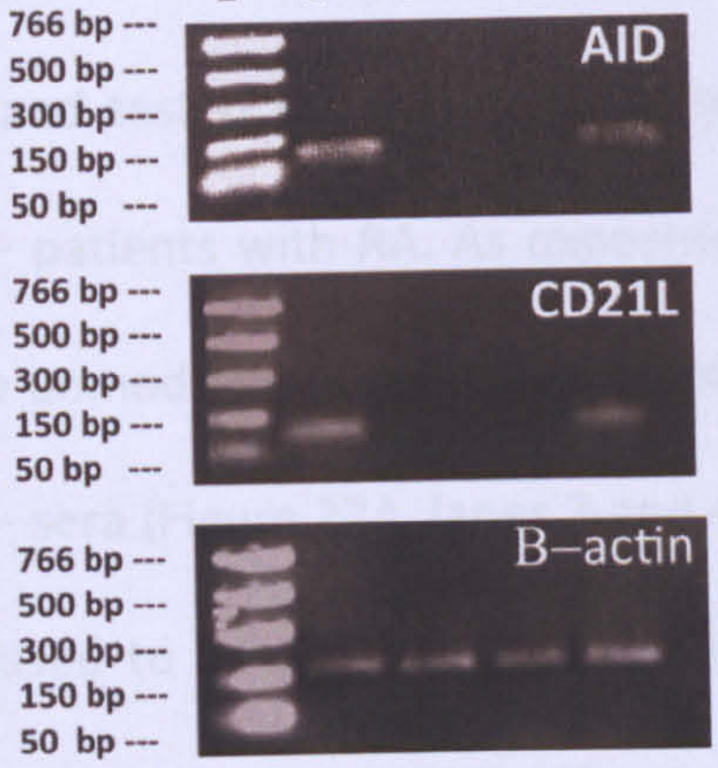
(C–F) CXCL13, LTβ, BAFF, and APRIL transcript analysis by QT-PCR. AID expression within synovial tissue was associated with the up-regulation of both CXCL13 and LTβ but no significant difference was seen in the levels of APRIL and BAFF. The lower and upper margins of the box represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as a horizontal line within the box. *p*-Values were calculated using the Mann-Whitney U test.

A

	AID +ve	AID –ve
CD21L +ve	12	0
CD21L -ve	0	13

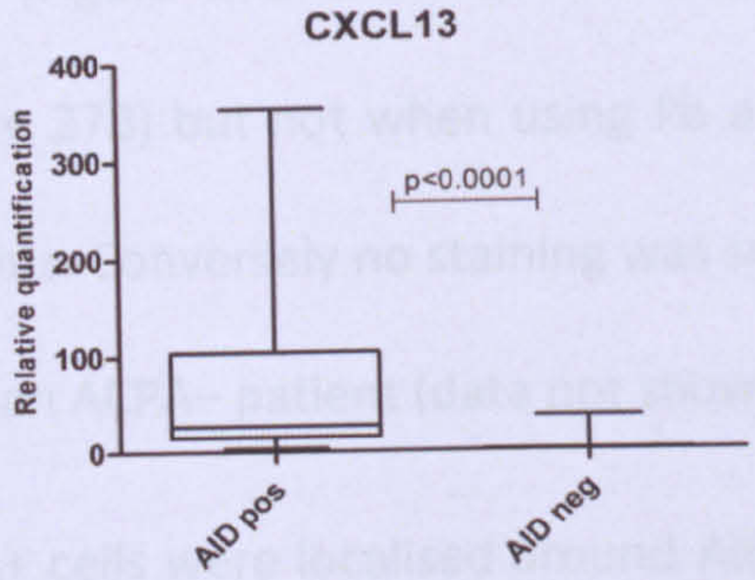
chi squared $p < 0.0001$

B

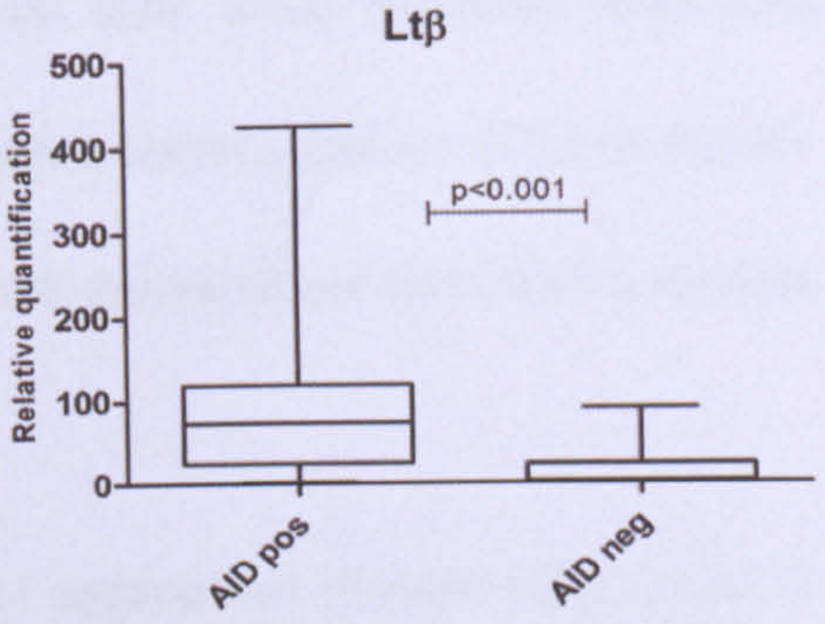


lane 1: PCR marker
lane 2: RA synovium AID,CD21L+ve
lane 3: RA synovium AID,CD21L -ve
lane 4: T cells
lane 5: lymph node

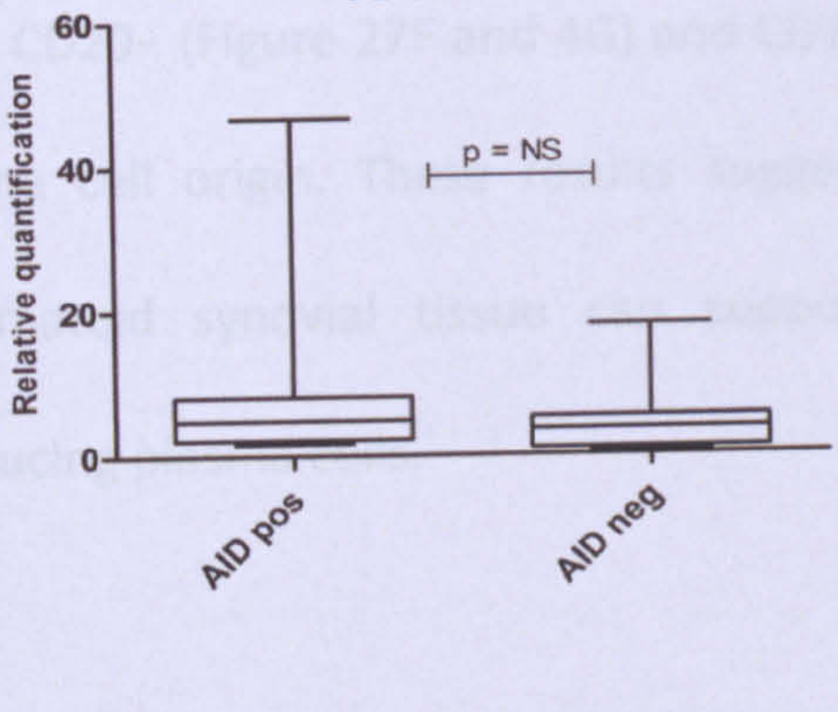
C



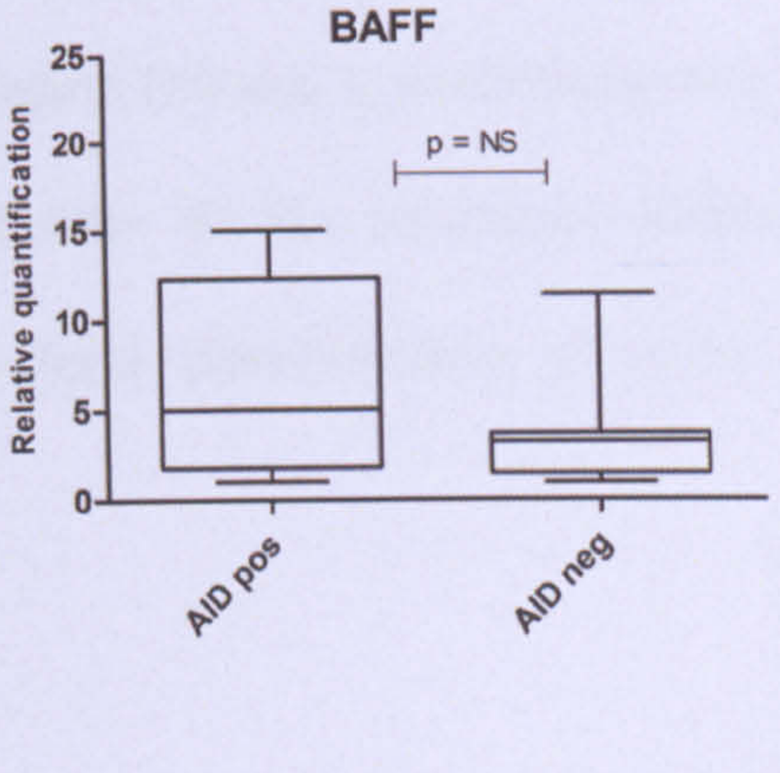
D



E



F



AID-Positive Follicles Are Surrounded by Plasma Cells Producing ACPA

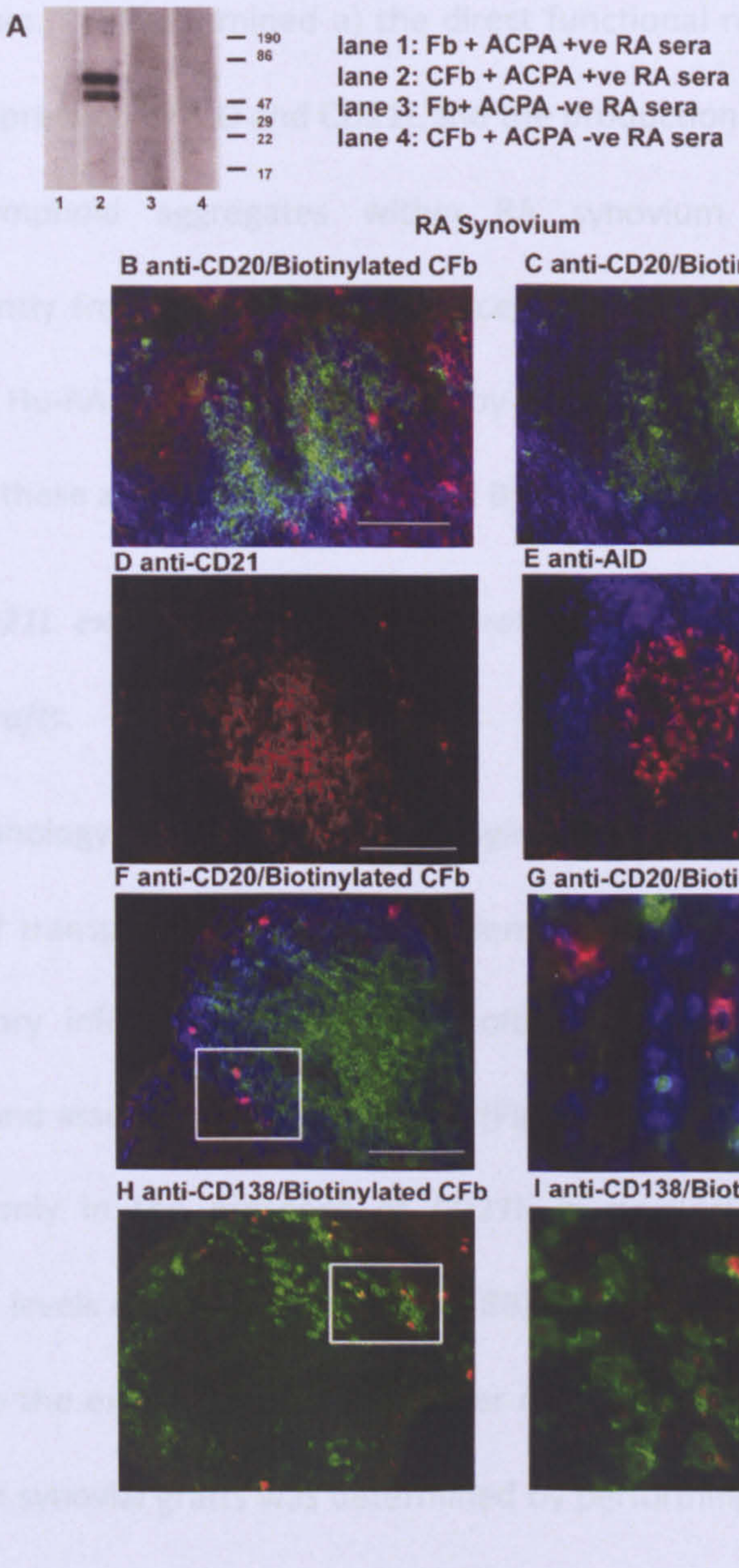
I next investigated whether AID+/FDC+ GC-like structures within the rheumatoid synovial membrane were associated with the in situ production of ACPA.

Biotinylated citrullinated fibrinogen (CFb) was produced as previously reported (386) and tested for specificity using pooled sera from three ACPA+ and three ACPA– patients with RA. As expected, ACPA+ sera showed reactivity to CFb but not to unmodified Fb (A, lanes 1 and 2), whilst no reactivity was observed with ACPA– sera (Figure 27A, lanes 3 and 4). Biotinylated CFb and biotinylated Fb were then used to detect in situ production of ACPA in sections double stained for CD20 (Figure 27B and 4C). Numerous positive cells were detected with CFb (Figure 27B) but not when using Fb as a negative control (Figure 27C) in ACPA+ patients. Conversely no staining was seen around germinal centres from a sample from an ACPA– patient (data not shown).

ACPA+ cells were localised around AID+/CD21+ aggregates (Figure 27D, E and F), were CD20– (Figure 27F and 4G) and CD138+ (Figure 27H and I), confirming their plasma cell origin. These results suggest that AID+ GC-like structures within rheumatoid synovial tissue can support the local differentiation of ACPA-producing plasma cells.

Figure 27: AID + Aggregates Are Surrounded by Plasma Cells Producing ACPA within Rheumatoid Synovium

(**A**) Immunoblot analysis of reactivity of pooled ACPA +ve and -ve RA sera to citrullinated fibrinogen (CFb) (lanes 2 and 4) and control non-citrullinated fibrinogen (Fb) (lanes 1 and 3). Two protein bands between 60-80kDa in size, corresponding to the α- and β-chains of CFb, are recognized by the ACPA+ve RA sera (lane 2) but not control Fb (lanes 1). ACPA-ve sera (lanes 3 and 4) show no immunoreactivity towards either citrullinated or control Fb. (**B-G**) Double or single immunofluorescence analysis showing phenotypic characterization and immunoreactivity towards citrullinated proteins within RA synovial tissue. Sequential sections of RA synovial tissue were double stained with anti-CD20 to detect B-cells (green) together with either biotinylated CFb (**B**, red) or control biotinylated Fb (**C**). Sections were counterstained with DAPI. Positive staining is only seen in the sections incubated with biotinylated CFb confirming the presence of anti-citrullinated protein immunoreactivity. (**D-G**) Sequential sections of rheumatoid synovium stained with anti-CD21 to detect FDC (**D**), anti-AID (**E**), double stained with anti-CD20 (green) and biotinylated CFb (red) (**F-G**) and double stained with anti-CD138 (green) to detect plasma cells and biotinylated CFb (red) (H-I). CD21 was visualized with vector red. AID, CD20, CD138 and biotinylated CFb were incubated with secondary Abs/streptavidin conjugated to ALEXA fluorochromes. AID and CD20 were counterstained with DAPI. ACPA Ab producing cells were seen scattered around FDC and AID+ve aggregates (**F**). ACPA+ve cells were consistently CD20-ve (**G**) (x60 magnification of F) and CD138+ve (**H**) (x60 magnification of G). (Original magnification x20 **B-F,H**). Scale Bars: 200μm (**D-F,H**), 50μm (**B,C**) 20μm(**G,I**)


A
190
86
47
29
22
17
1 2 3 4
lane 1: Fb + ACPA +ve RA sera
lane 2: CFb + ACPA +ve RA sera
lane 3: Fb+ ACPA -ve RA sera
lane 4: CFb + ACPA -ve RA sera
RA Synovium
B anti-CD20/Biotinylated CFb
C anti-CD20/Biotinylated Fb
D anti-CD21
E anti-AID
F anti-CD20/Biotinylated CFb
G anti-CD20/Biotinylated CFb
H anti-CD138/Biotinylated CFb
I anti-CD138/Biotinylated CFb

The histomorphological analysis described above suggested that ectopic AID+/CD21+ aggregates are capable of sustaining in situ ACPA production in the RA synovium. I next examined a) the direct functional relationship between the synovial expression of AID and CD21L and the production of ACPA and b) whether ectopic lymphoid aggregates within RA synovium maintain functionality independently from incoming immune cells from the periphery. To this end, I generated Hu-RA SCID mice chimeras by implanting fragments of RA synovial tissue into these animals (Figure 28A and B), and analysed the results at 4 wk.

(i) AID/CD21L expression and B cell proliferation are maintained within RA synovial grafts.

Histomorphology and immunohistological analysis of paraffin-embedded sections of transplanted RA synovium demonstrated viable grafts with residual inflammatory infiltrates (Figure 28C), often characterized by persistent FDC networks and associated AID expression (Figure 28D and E). Importantly, AID was detected only in the presence of CD21L in synovial grafts and AID mRNA expression levels closely correlated (r=0.88) with those of CD21L (Figure 28G). In addition to the expression of AID, further evidence of functional activation of B cells within synovial grafts was determined by performing double staining for the B cell marker CD20 and Ki67, a marker of cell proliferation. Double stained, proliferating B cells were identified within aggregates within grafts at 2 wk (Figure 28F), confirming that B cells within synovial grafts are able to both survive and proliferate in this micro environment, and hence, retain the capacity to function as GC B cells.

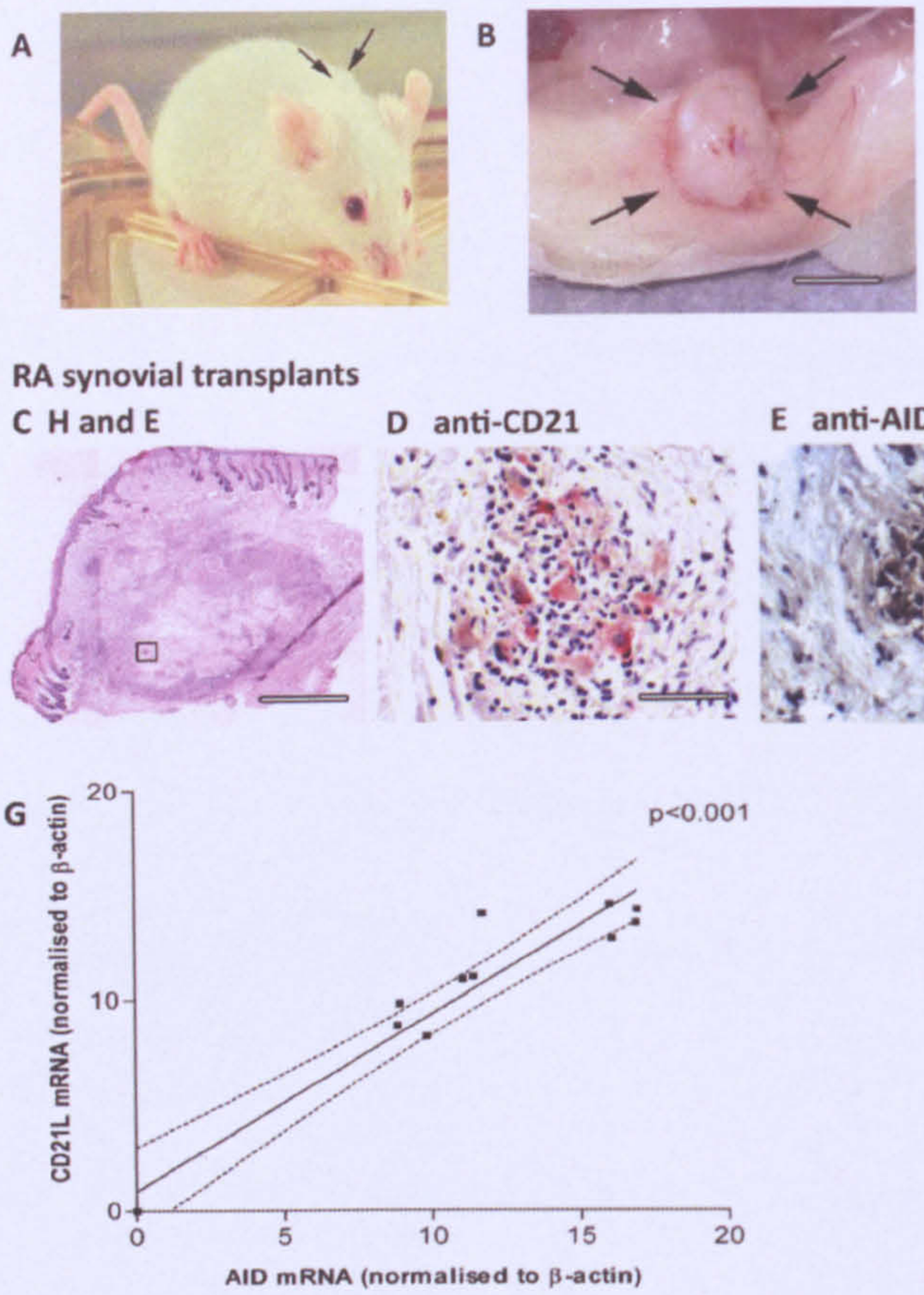

A
B
RA synovial transplants
C H and E
D anti-CD21
E anti-AID
F anti-CD20/anti-Ki67
G
p<0.001
CD21L mRNA (normalised to β-actin)
AID mRNA (normalised to β-actin)
APRIL
BAFF
TNFα
H
I
J
p<0.05
p = NS
p<0.0001
Relative quantification
AID pos
AID neg
Ltβ
CXCL13
K
L
p<0.05
p<0.05

Figure 28: Rheumatoid synovial grafts in SCID mice maintain AID expression and the upregulation of genes determining lymphoneogenesis.

Rheumatoid synovial tissue (n=56) was transplanted into SCID mice (**A**) (arrows depict site of transplants). (**B**) Transplanted RA synovial tissue in SCID mouse. (**C**) Montage image of a paraffin embedded H and E stained section of transplanted tissue depicting the whole graft (original magnification x4, area outlined in black equates to aggregate seen at higher magnification in D and E). Sequential sections of transplants stained using conventional IHC for CD21 (**D**), AID (**E**) and double immunoflouresence for Ki67 (green) and CD20 (red) (**F**) (inset x40 magnification arrowheads indicating cells staining positively for Ki67 and CD20) demonstrated persistent FDCs, associated expression of AID (original magnification x40) and continued proliferation of B cells. Four weeks post-transplantation, total RNA from the grafts was reverse transcribed and used for TaqMan quantitative real time-PCR (QT-PCR) to determine the mRNA expression levels of AID and CD21L and a number of genes known to correlate with lymphoid tissue organization. (**G**) A significant correlation between levels of transcripts for AID and CD21L was seen and AID was detected only in the presence of CD21L. Tissues were stratified according to the presence or absence of AID as determined by QT-PCR and analysed for the expression of the selected cytokines/chemokines genes between the groups. A significant difference is seen in levels of APRIL (**H**), CXCL13 (**I**), TNFα (**J**) and LTβ (**K**). Significant p values are depicted, Mann-U Whitney test. No significant difference is seen between levels of BAFF (**L**). The lower and upper margins of the box represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th

percentiles, respectively. The median is shown as a horizontal line within the box. Scale Bars: 6mm (**B**) 2mm (**C**), 50μm (**D,E,F**)

(ii) AID and CD21L expression in RA synovial grafts is associated with the up-regulation of genes regulating ectopic lymphoneogenesis.

To evaluate whether the sustained expression of AID and CD21L mRNA in RA synovial grafts was associated with dynamic gene expression regulating ectopic lymphoneogenesis, I analysed mRNA expression levels of CXCL13, LTβ, TNFα, APRIL, and BAFF. As shown in Figure 28H-K, expression levels of the TNF family members LTβ, TNFα, and APRIL, as well as the lymphoid chemokine CXCL13 were significantly higher in AID+ RA grafts as compared to AID− grafts. Conversely, although BAFF was abundantly expressed in all RA synovial grafts, no significant differences between AID+ and AID− grafts were found. These results are in keeping with previous reports (644), showing no difference in the expression patterns of BAFF between CD21+ and CD21− synovial tissues, but an up-regulation of APRIL mRNA in GC+ synovitis, and indicate that ectopic lymphoneogenesis in RA synovium develops and is maintained in the presence of high expression levels of these factors.

(iii) AID and CD21L expression in RA synovial grafts is associated with ongoing CSR and in situ production of ACPA antibodies.

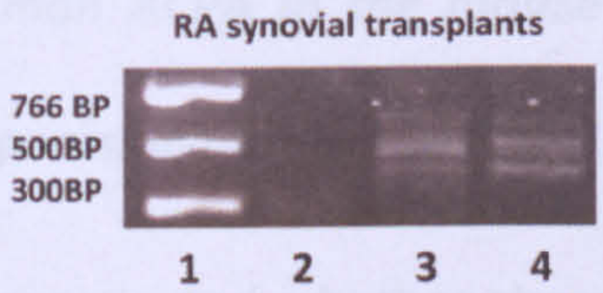
In order to confirm that AID expression in RA synovial grafts was functional, evidence of ongoing CSR was sought by analysing for the expression of circular transcripts. Circular transcripts are known to be specifically associated with AID expression and exclusively detectable in B cells undergoing CSR, but not by

plasma cells, which have already undergone CSR (201). By performing RT-PCR for Iγ-Cμ circular transcripts, which are transiently produced for around 48 h following class switching from IgM to IgG (201), I demonstrated (Figure 29A) their invariable detection in those RA transplants also expressing AID and CD21L mRNA, confirming the functionality of AID expression in synovial grafts.

Figure 29: ACPA production in the HuRA-SCID mouse is associated with functional AID expression within synovial grafts

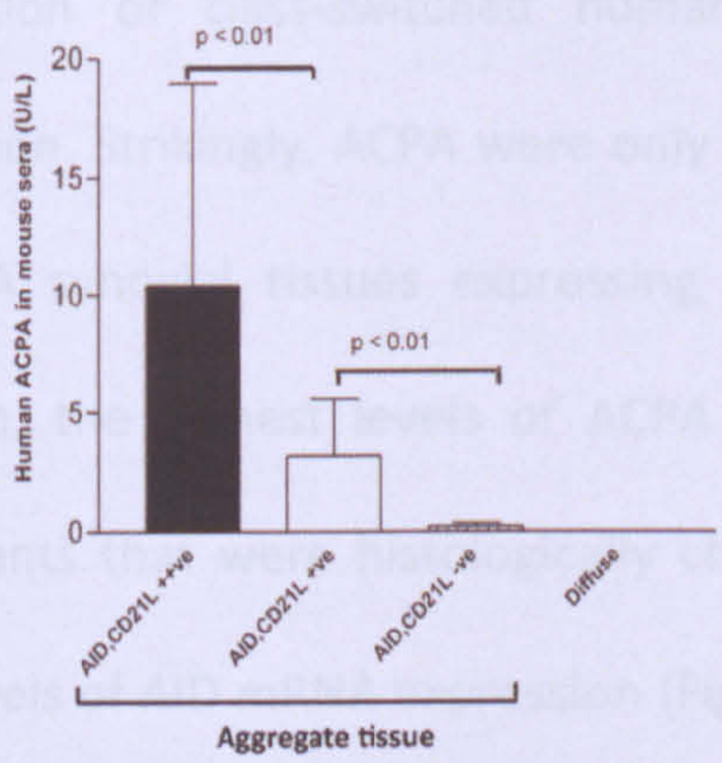
Human RA synovium was transplanted into 34 SCID mice. Four weeks post-transplantation mice were sacrificed and all sera tested for the presence of ACPA, while mRNA from the grafts was reverse transcribed and used for TaqMan quantitative real time PCR (QT-PCR) to determine the number of transcripts for AID and CD21L and for RT-PCR to determine the presence of circular transcripts. (**A**) 1.8% agarose gel showing representative PCR products from RT-PCR performed for Iγ-Cμ circular transcripts (double bands representing alternatively spliced transcripts and confirming specificity (698)) indicating ongoing class switch recombination (CSR) in synovial grafts positive for AID,CD21L and producing ACPA (lane 3 and 4) but not in transplants negative for AID,CD21L (lane 2). (**B**) Synovial transplants from 6 RA patients were grouped according to histological classification, as aggregate vs diffuse and AID, CD21L mRNA expression as high (++), low (+) and negative (-). Significantly higher levels of ACPA were seen in those mice transplanted with synovium expressing high mRNA levels of AID, CD21L. Notably, ACPA were not seen in the absence of AID. (**C**) To establish the relationship within the same patient between levels of AID expression and ACPA production, synovial grafts from an illustrative patient (P1) were stratified according to the presence or absence of AID as determined by QT-PCR (**D**) (horizontal line indicates cut off for positivity). Circulating ACPA from synovial grafts were produced at a significantly higher level in AID+ve compared to AID-ve grafts. Error bars indicate mean levels of ACPA + SD. P values were calculated using Kruskal-Wallis test (B) and Mann Whitney U test (C)

A

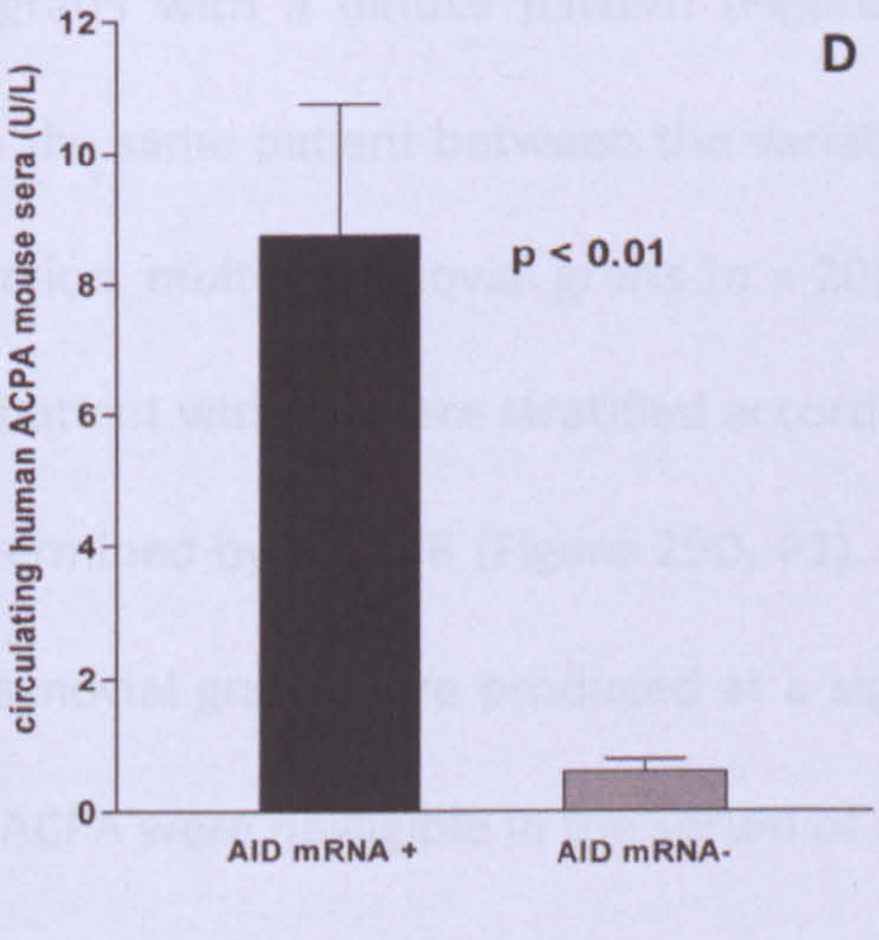


1 PCR marker
2 AID, CD21L negative
3,4 AID, CD21L positive

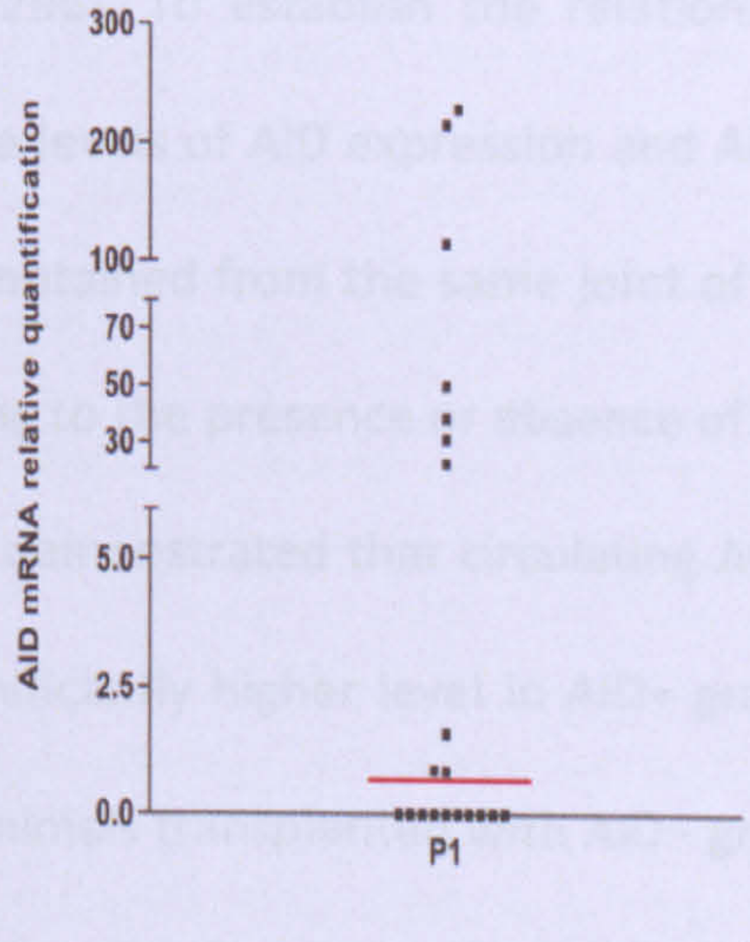
B



C



D



(iv) Human ACPA in the mouse circulation are associated with intra-graft expression of AID and CD21L mRNA.

Finally, I investigated whether there was a direct relationship between AID and CD21L mRNA expression within transplanted RA synovial grafts and the production of class-switched human IgG ACPA detectable in the mouse circulation. Strikingly, ACPA were only detected in the sera of mice transplanted with RA synovial tissues expressing AID and CD21L mRNA (Figure 29B). In addition, the highest levels of ACPA in the serum were detected in the RA transplants that were histologically characterized by an aggregate pattern and high levels of AID mRNA expression (Figure 29B). The levels of ACPA decreased in line with the decrease of AID mRNA expression, whilst no ACPA were detected in AID– grafts with a diffuse pattern (Figure 29B). To establish the relationship within the same patient between the variable levels of AID expression and ACPA production, multiple synovial grafts (n = 20) obtained from the same joint of the same patient with RA were stratified according to the presence or absence of AID as determined by QT-PCR (Figure 29D, P1). I demonstrated that circulating ACPA from synovial grafts were produced at a significantly higher level in AID+ grafts, while ACPA were negligible in the serum of animals transplanted with AID– grafts (Figure 29C). These data indicate that, despite the known variability between different synovial biopsies within the same patient, a functional relationship between AID expression and ACPA production is maintained.

Discussion

In this report I provide strong evidence that lymphoid structures in the target organs of a human autoimmune disease are functional by demonstrating that within the synovial membrane of patients with RA, ectopic lymphoid aggregates characterized by FDC networks invariably express AID and are surrounded by ACPA producing plasma cells. In addition, using the human RA-SCID mouse chimera model, I demonstrate that transplanted RA synovial grafts containing ectopic lymphoid structures support B cell survival and proliferation, maintain AID expression, continuously promote ongoing CSR and produce human IgG ACPA. This phenomenon, characterized by the sustained high expression levels of genes regulating ectopic lymphoneogenesis and in the absence of new influx of immune cells, indicates that the establishment of follicular structures in the synovium can lead to self-perpetuating autoimmunity. The self-sustained survival of B cell niches, within rheumatoid synovium, may also have a critical impact on the capacity of B cell depleting biologics to modulate chronic inflammation and autoimmunity.

The elucidation of the mechanisms coupling chronic inflammation and the production of disease specific autoantibodies within RA synovium is of pivotal importance because ACPA are highly disease specific markers of RA, are strongly associated with a more destructive arthritis and are independent predictors of a poorer prognosis (529;530). However, despite recent evidence that ACPA can mediate tissue damage in animal models (481), confirmation of a direct pathogenic role for ACPA in patients with RA is currently lacking. The presence of

ectopic GC-like structures, characterized by T/B cell aggregates and CD21+ FDC networks, in the synovium of a subset of patients with RA has long been known. These structures have been associated with the expression of cellular and molecular markers associated with GC responses in secondary lymphoid organs (217;334;673) and analysis of Ig sequences from microdissected GCs from synovial tissue has demonstrated hypermutated and clonally related sequences (415), suggesting that an antigen driven B cell response is ongoing within RA synovium. Nonetheless, direct evidence that ectopic lymphoid tissue in RA synovium contains the required molecular machinery to sustain ongoing CSR and affinity maturation of autoantibodies has so far been lacking. Recent evidence has demonstrated that the processes of SHM and CSR are critically dependent on the expression of the enzyme AID (197) and that AID expression is exclusively associated with B cells undergoing SHM and CSR (700). Therefore delineating its expression pattern has allowed us to establish that B cells activate the molecular machinery responsible for production of affinity matured antibodies in follicular structures within the RA synovial membrane.

In this study I provide, to my knowledge, the first demonstration that AID is invariably expressed within rheumatoid synovial T/B cell aggregates containing CD21+ FDC networks, with a distribution closely recapitulating that seen in secondary lymphoid organs, providing direct evidence that ectopic GC-like structures represent a functional tertiary lymphoid organ capable of activating the molecular machinery necessary to sustain SHM and CSR within the synovial membrane. Accordingly, ongoing CSR, as demonstrated by the expression of circular transcripts, was invariably detectable within AID+/FDC+ RA synovial

tissues. These results confirm and expand our recent observations in Sjögren's syndrome (675) in which FDCs appeared necessary for sustaining AID expression in the ectopic lymphoid tissue found in salivary glands, where an antigen-driven B cell response can occur (701). This evidence, further supported by my data demonstrating the presence of AID transcripts only in the presence of CD21L mRNA, strongly supports the conclusion that within the rheumatoid synovial membrane, AID requires the presence of FDCs for expression. I also provide further histological evidence supporting both the functionality of these AID+ structures and their involvement in the production of ACPA by showing that these structures are surrounded by ACPA producing plasma cells.

By evaluating the expression of CD21L and AID mRNA using QT-PCR, a sensitive and highly specific method, I were able to identify functional sites of ectopic lymphoneogenesis in about 50% of the RA synovial samples. This prevalence was significantly higher than that observed using histological analysis and semi quantitative PCR reported in approximately 25% (217), and is in line with a recent observation by other groups (317). These additional (low) CD21L+ tissues were always of aggregate appearance, and were invariably accompanied by AID expression and ongoing CSR, strongly supporting the concept that such aggregate structures are also functional. These results demonstrate that the presence of lymphoid aggregates and ongoing GC reactions in RA synovium is a phenomenon considerably more common than has recently been proposed (388;416;426).

In this report, by using the HuRA-SCID mouse model I was able to provide direct evidence of functionality and to present a number of novel findings regarding the

production of ACPA within the rheumatoid synovial membrane. First, as in the original RA synovial tissue, the expression of AID within the transplanted tissue was consistently associated with the presence and levels of CD21L. This, in turn, was invariably associated with ongoing CSR and the production of human IgG ACPA detectable in the mouse sera. In addition, levels of AID within the RA transplants directly correlated with the levels of ACPA in the mouse sera. Finally, even low-level expression of AID and CD21L within the transplants was still associated with significant ACPA detection in the sera, while ACPA were barely detectable in the absence of AID mRNA expression in the RA grafts. Overall, these results strongly support the notion that lymphoid structures expressing AID and CD21L can directly contribute to ACPA production within the rheumatoid synovial membrane. Indeed, as further evidence of AID functionality within synovial tissue, I demonstrated that AID expression supports the expression of Iγ-Cμ circular transcripts in the RA synovial grafts, and hence the presence of ongoing class-switching from IgM to IgG. Because circular transcripts are only transiently produced in association with CSR (201), this demonstrates that class-switching is still ongoing within the RA synovial grafts, a result in line with the detection of human IgG ACPA in the mouse circulation. It is important to emphasise that these experiments cannot formally exclude the possibility that pre-existing long-lived plasma cells, either generated in situ or migrated from secondary lymphoid organs, might also contribute to the production of ACPA in RA synovium and in transplanted animals. Despite this limitation, the topographical proximity of ACPA-producing plasma cells to AID+ follicular structures and the close association between the level of ACPA production in the mouse serum and higher

levels of AID within the same tissue strongly suggest that autoreactive plasma cells can be generated within ectopic lymphoid tissue. This concept is in agreement with the significantly higher levels of synovial ACPA recently demonstrated in synovial tissues containing lymphoid aggregates (424). The lack of detection of GC CD20+ B cells reacting with citrullinated fibrinogen is in line with previous reports showing that reactivity toward labelled autoantigens is typically restricted to plasma cells surrounding ectopic GCs (386;702), and is in all probability due to the low density of antigen-specific antibodies on the cell surface of CD20+ B cells compared to the much higher quantity of high-affinity antibodies produced by plasma cells as they migrate out of GC. Finally, although the evidence that I provide is highly suggestive of a direct role for AID+ lymphoid structures in generating in situ autoantibody-producing cells, a formal demonstration would require evidence, i.e., in the SCID-HuRA model, that disruption of FDC networks abrogates plasma cell differentiation and ACPA production.

A final and highly relevant observation obtained in the HuRA-SCID chimera is that the RA synovium, in the presence of follicular structures expressing AID and CD21L, behaves as a self-sustained microanatomical unit of ectopic lymphoid tissue. Furthermore, within the time frame analysed, the RA synovium supports the proliferation of B cells and maintains an intrinsic ability to function as an autoantibody-producing site independent of circulating immune cells. The continued expression of AID and CD21L and the association with ongoing CSR and ACPA production in this environment support a key role for ectopic lymphoid tissue within the synovial membrane in driving the ongoing disease process, and

may also explain the lack of direct correlation between peripheral B cell depletion and clinical response to the anti-CD20 monoclonal antibody rituximab (703;704). The persistence of ectopic lymphoid structures in RA synovial tissues following transplantation into SCID mice is likely to be related to the sustained overexpression of genes regulating ectopic lymphoneogenesis and autoreactive B cell survival, such as the TNF family members LTβ, TNFα, and APRIL, as well as the lymphoid chemokine CXCL13 (217;334). LTβ, TNFα, and CXCL13 were all significantly more highly expressed in AID+/CD21L+ as compared to AID−/CD21L− RA tissues, and high expression was maintained in RA synovial grafts displaying AID, ongoing CSR, and ACPA production. I suggest that the up-regulation of these key cytokines within RA synovial tissue allows differentiation and maintenance of FDC networks within T/B cell aggregates to form functional microanatomical immunological units, and hence, allow the up-regulation of AID and subsequent production of ACPA. In addition, my results might also explain the fall in ACPA levels resulting from the dual blockade of TNFα and LTα with etanercept (705), and the blockade of TNFα by infliximab (491) in patients with RA.

In conclusion, my work demonstrates that ectopic GC-like structures are not only functional in rheumatoid synovitis, but that their presence may contribute to disease pathogenesis via the production of ACPA. These data elucidate the mechanism of production of ACPA in the synovial membrane and thereby provide evidence of a pivotal role for AID in the pathogenesis of RA. I therefore propose that AID may be targeted for the development of novel therapeutic agents.

Chapter 6: The Identification and Characterisation of Interfollicular Large B cells within the RA Synovial Membrane

Introduction

B cell maturation begins in the bone marrow where B cell precursors undergo rearrangement of their variable (V), diversity (D) and joining (J) segments under the control of recombination activating genes (RAG) genes. The joining of these V, D and J segments is accompanied by the generation of random-linking N segments, under the control of terminal transferase. Naive B cells (carrying IgM and IgD as their surface antigen receptor) emerge from the bone marrow carrying inherent diversity within their antigen binding regions of antibody. Further diversity within the antigen binding sites of surface immunoglobulin (Ig) is then introduced following entry into germinal centres (GC) within lymphoid tissue. This diversity is a result of a process of somatic hypermutation (SHM), a process critically dependent on the enzyme activation induced cytidine deaminase (AID)(197). Mutations introduced that confer increased binding affinity of antigen for antibody result in escaped apoptosis and, either re entry to the GC reaction to undergo further cycles of SHM, or final differentiation into plasma or memory cells. B cells have thus been traditionally classified into naive and GC/post GC B cells, dependent on the pattern of somatic mutations within the V region segments of the Ig heavy chain (H). Additional diversity in effector functions of antibodies are introduced as result of a process of class switch recombination (CSR), again critically dependent on AID(197).

A report in 2003 by Marafioti et al (5) identified a novel B cell subtype in association with GCs within lymphoid tissue, that of Interfollicular (IF) large B cells. These cells were characterised by a large dendritic like morphology, a mutated IgH and a location within T cell rich interfollicular areas. This cell type had been previously reported in mice as extrafollicular plasmablasts. The authors

of this paper suggested these cells were post GC B cells, due to their mutated IgH, though noted them to be distinct from the classical post GC plasma and memory B cells, lacking in particular the markers CD27 and CD138. Although the dendritic phenotype of these cells suggested a role in antigen presentation, this was not supported by the lack of expression of the co stimulatory molecules such as CD80 and CD86 and thus their immunological role was uncertain. In addition no single marker was found to be capable of reliably identifying these cells from other cells such as monocytoid or MZ B cells, complicating their identification within other tissues. This work was immediately followed by data from another group demonstrating that IF large B cells were the only cell outside of GCs to express AID(196). As AID is critically required for both CSR and SHM its association with a cell previously noted to have a somatically mutated IgH locus raised the possibility that IF large B cells may represent an extrafollicular antibody response, rather than originating and mutating their IgH within the environment of the GC. This report confirmed ongoing AID activity via the detection of circular transcripts and thus ongoing CSR in IF large B cells, but did not re examine the IgH for SHM. The presence of ectopic GC within the RA synovial membrane has been acknowledged for some time and evidence suggests a number of similarities with GCs within secondary lymphoid tissue including the detection of follicular dendritic cell networks (FDC) networks, high endothelial venule expression, T/B cell segregation(334;673) and ongoing clonal B cell proliferation(415;423). I had previously noted the presence of CD20+, IgD- B cells surrounding ectopic GC within RA synovial tissue, and the large dendritic phenotype of a cohort of these cells suggested that ectopic GCs within RA synovial tissue may be capable of supporting IF large B cells. Although the detection of IF large B cells within

ectopic lymphoid tissue had not previously been reported, their identification particularly within RA synovial tissue was thought to be of potential clinical relevance with the suggestion that they represented likely precursors for diffuse large B cell lymphomas(196), the most common malignancy associated with RA(706).

Aims and objectives:

The aims of this chapter were to:

1. Determine whether a cohort of CD20+, IgD- large B cells surrounding ectopic GC represented the recently described novel IF large B cells
2. Determine whether extrafollicular AID expression within IF large B cells within lymph nodes and within the RA synovial membrane is associated with a somatically mutated IgH.

Materials and Methods

Patients and samples

Synovial tissue was collected from 21 RA patients following arthroplastic joint surgery. All patients fulfilled the 1987 revised ACR guidelines for RA. Paraffin embedded tissue was available from all patients and from 10 patients additional samples were available that had been snap frozen in O.C.T. Normal human lymph nodes, obtained from 2 patients undergoing vascular surgery were also available. In addition from 3 patients with Sjogren's syndrome, formaldehyde fixed paraffin embedded tissues were obtained from the minor salivary glands of 2 patients and from the parotid gland of 1 patient, all known to contain FDC+ lymphocytic aggregates. All patients fulfilled the revised criteria of the American-European consensus group(707) for either primary (pSS) or secondary (sSS) Sjogren's syndrome. All procedures were performed following written informed consent approved by the hospital's ethics committee (REC 05/Q0702/1). Demographic and main laboratory characteristics of RA patients are described in Table 18.

Table 18: Demographic and laboratory characteristics of the RA patients enrolled in this study

RA patients	
Number of patients	21
Age (y) mean ± SD	51 ±10
Female (%)	74
Disease duration (y) mean ± SD	16 ± 13.5
Rheumatoid factor+ (%)	76
Aggregate (CD21 network+) tissue (%)	24

Grading analysis, histological characterization of lymphoid proliferation and AID expression in RA synovial tissue

As IF large B cells had been identified in lymphoid tissue in association with GCs all 21 synovial tissue samples were assessed for degree of lymphoid organisation. 5μm sections were cut sequentially from 3 levels of each paraffin block 50μm apart. Following routine H&E staining synovial tissue was classified as aggregate or diffuse according to the predominant lymphocytic infiltrate, as previously reported(421). Sequential sections of synovial tissue characterised as aggregate then underwent double staining for the markers CD21 and Bcl2, identifying FDCs and GC mantle zone B cells respectively. Following antigen retrieval with Target retrieving solution (DAKO) and proteinase K digestion(6 minutes incubation), slides were sequentially incubated overnight with Bcl2 (1:50 dilution) and CD21 (1:20 dilution), followed by appropriate secondary antibodies and developed with DAB and vector red respectively. Sequential sections of synovial tissue also underwent double staining for CD20 (1:20 dilution) and IgD (1 in 80 dilution) with one hour primary antibody incubations and followed by the Dako EnVision Doublestain system (DakoCytomation) used according to manufacturer's instructions. Frozen sections of aggregate synovial tissue also underwent double immunofluorescent staining for CD20 and IgD in order to confirm the results of conventional IHC. Finally sequential sections were also incubated with CD3 (1 in 50 dilution) for one hour and following incubation with biotinylated goat anti-rabbit Ig developed with DAB, in order to identify T cells. The presence of ectopic GCs was determined by the presence of lymphocytic aggregates with FDC networks and T/B cell segregation.

Staining for AID was performed on sequential sections of all 21 RA synovial tissue samples in order to determine whether IF large B cells could be identified within RA synovial tissue and to determine whether they were found solely in association within ectopic GC. After Ag unmasking using Target retrieval solution (pH 6; Dako) and incubation with protein block solution (Dako) primary Ab EK2-5G9 was incubated (1:20 dilution) for one hour at room temperature and biotinylated rabbit anti-rat Ig used as a secondary Ab. Following a1h incubation and three washes, HRP or AP-streptavidin-biotin complex (Dako) was added to the section and incubated for 30 min. After further washes colour reaction was developed with diaminobenzidine (HRP,DAB; Dako) or Vector red (for AP; Vector Laboratories). Double staining for AID and the cellular markers CD3 and CD20 was also performed in order to determine the cellular source of AID+ cells.

AID expression within minor salivary glands and parotid tissue of Sjogren's syndrome patients

In order to determine whether IF large B cells were specific for RA synovial tissue, samples of another chronically inflamed tissue known to acquire features of ELN, the salivary glands of Sjogren's patients(383), was used as a control tissue. 3 tissue samples known to contain FDC+ lymphocytic aggregates were identified from the tissue bank. The cellular infiltrate and degree of lymphoid organization were assessed by immunohistochemical staining of sequential sections of minor salivary glands with Abs to CD3, CD20 and CD21, as previously reported (11, 12). Briefly after de-waxing and re-hydration formalin fixed, paraffin-embedded 3μm thick sections underwent appropriate Ag retrieval with Target retrieval solution or proteinase K (DakoCytomation). Double staining for CD3 and CD20 was used to analyze T/B cell segregation using the Dako EnVision Doublestain

system (DakoCytomation) as previously reported (11, 12). Sections sequential to those identified as expressing CD21 and T/B cell segregation underwent staining for AID as per synovial protocol although including a step to block endogenous biotin with the avidin biotin blocking system (DakoCytomation).

Single cell microdissection of IgD+, IgM+ and AID + B cells from lymph node

In order to determine whether AID+ IF large B cells expressed a somatically mutated IgH 10μm thick sections of frozen lymph node were cut for subsequent laser capture microdissection (LCM). Sections were mounted onto PALM membrane slides (ZEISS) in order to maintain morphological integrity of the tissue specimen. Slides were then covered with silver foil and stored at -80^{0}C until use. Prior to staining slides were defrosted in silver foil for 10 minutes before undergoing immunohistochemical staining for IgD and AID. Once defrosted tissue sections underwent fixation in 95% acetone at 4^{0}C for 10 minutes. Tissue was then incubated with protein block solution (DAKO) and tissue undergoing staining for AID was additionally incubated with 20% rabbit serum (DAKO) for 30 mins. This step was followed by incubation with primary antibody (Table 5). IgD was incubated at a dilution of 1:80 for 30 minutes and AID 1:20 for 1hour. Following two washes with TBS buffer, tissue for AID staining was incubated with rabbit anti-rat biotin secondary antibody (Table 5) for 30 mins and following two TBS washes with ABC HRP (DAKO) and developed with DAB solution (DAKO). IgD tissue was subsequently incubated with ABC-HRP (DAKO) and developed with DAB (DAKO). Following counterstaining with haematoxylin slides were washed for 5 minutes in tap water and then left to air dry overnight. Slides were then stored in an air tight container until needed.

Naive IgD+ cells carrying an unmutated IgH have been reported to result in a higher amplification efficiency than cells carrying a mutated IgH(5), so in order to establish experimental technique and determine efficiency of amplification of the IgH chain locus, single IgD+ cells were used for initial experiments. Single IgD+ cells were identified surrounding GCs within sections of lymph node and underwent LCM followed by pressure catapulting using a PALM microbeam system (ZEISS). Specimens were collected onto the caps of PCR tubes specifically developed for increasing retention of tissue due to a specialised adhesive coating on the cap (PALMAdhesive cap200 opaque). Each cap was prefilled with 20µl of proteinase K buffer (made up of 10µl of 2.5mg/ml proteinase K stock solution, 10µl of Taq DNA polymerase 10x buffer (promega) and 80µl of dH_2O(BDH)). Tubes were attached to the caps and kept inverted, to ensure catapulted cells remained within the proteinase K solution for digestion. Inverted tubes were then transferred to a hybridising oven preheated to 56^0C (pre saturated with distilled water), for a 1h digestion. Proteinase K was subsequently inactivated by heating to 95^0C for 10 mins in a PCR machine. Tubes were then placed on ice and spun in a microfuge at 13000 rcf for 5 mins and kept at 4°C until use.

Although previous reports had noted a success rate of 60% when amplifying IgH from single cells, in order to determine optimum sampling efficiency within our laboratory between 1 and 20 singly microdissected cells were catapulted into each cap. In addition, as a positive control, IgM+ peripheral blood mononuclear cells (PBMCs) were sorted using a fluorescence activated cell sorter (FACSCaliber) into single and groups of 10 cells. Once sampling efficiency was determined, AID +ve IF large cells were microdissected from lymph node.

Amplification of IgH chain from microdissected cells

IgH chain genes were amplified using a semi nested PCR approach with internal and external J_H family primers as previously described(708;709). For the first amplification step FR1 primers for the V_H family members and J_H primers were both used at a concentration of 50ng/50µl of PCR reaction, with 20mM dNTPs to a final concentration of 0.2mM, 1x Taq buffer (Promega), and 25mM $MgCl_2$ to a final concentration of 2mM made up to a final volume of 28µl per reaction with $_dH_2O$. 28µl of mastermix was then added to each tube containing the digested microdissected cell and centrifuged for 1min at 1000rpm. DNA was then denatured for 10mins at 95 ^{0}C and the PCR conditions were as follows cycle 1; 7 cycles of denaturation for 1 min at 94 ^{0}C, annealing for 2 mins at 64 ^{0}C, and extension for 45s at 72 ^{0}C, cycle 2; 39 cycles of denaturation for 30s at 94 ^{0}C, annealing for 30s at 52^{0}C and extension for 90s at 72^{0}C. The mastermix for the second step of the PCR was made up with same final concentrations of dNTPs, Taq buffer and $MgCl_2$, but with both V_H and J_H primers at a concentration of 100ng/50µl PCR reaction. On completion of the first PCR 3µl of PCR reaction was then added to 45µl of mastermix and the second PCR programme run as the first round however with an annealing temperature of 66^{0}C in the first cycle and a reduction to 35 cycles and for cycle 2 an annealing temperature of 54^{0}C. PCR products were then run on a 1.8% agarose/TBE gel to check size of PCR product (424bp).

DNA was then purified and ligated into pGEM T-Vector plasmids (Promega). JM109 High Efficiency Competent cells (Promega) were then transformed and plated onto LB/ampicillin/IPTG/X-gal plates. White colonies were then picked off and PCR performed for specific markers of the β-galactosidase gene (M13

primers), allowing PCR using M13 primers to detect clones with correct size insert (expected band size 550bp). Stab colonies were then sent off for sequencing. Sequences were analysed using GeneJockey, and examined for evidence of somatic hypermutation.

Results

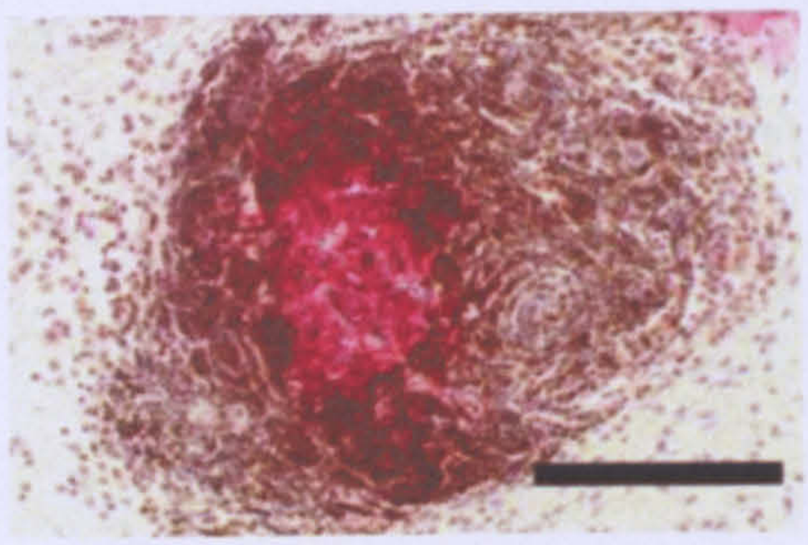
AID Identifies Interfollicular Large B Cells within the Rheumatoid Synovial Membrane in Association with FDC+ Aggregates

Ectopic GC with FDC networks (Figure 30A) and T/B cell segregation (Figure 30B-C) were identified in 24% (5/21) of RA patients analysed. GCs were consistently surrounded by large CD20+ IgD-ve cells identified using IHC (Figure 30C-D) often with a dendritic morphology (Figure 30E). In order to confirm single positivity for CD20, frozen sections of RA synovium underwent Immunofluorescent staining for CD20 and IgD (Figure 30F). Numerous single CD20+ cells were confirmed to surround ectopic GC. Such CD20+, IgD-ve cells had a dendritic like appearance and morphologically resembled IF large B cells (Figure 30D-E).

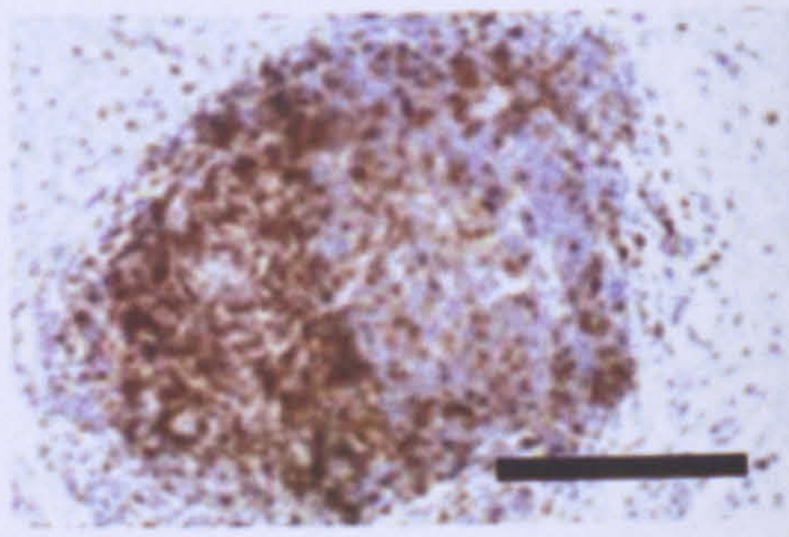
In order to specifically characterise these cells as IF large B cells I performed IHC staining for AID. In addition to AID+ B cells within FDC+ networks (described in Chapter 5), a second set of cells within the synovial membrane expressed AID outside this compartment. These cells were characterised by a large cytoplasm, often with a dendritic morphology, and were localized in close association with neighbouring T cells exclusively in CD21+ aggregates (Figure 31A and B). Using double immunofluorescence for AID and CD20 (Figure 31C and D), I confirmed that these AID+ cells were of B cell origin. The morphological appearance, tissue distribution, and predominant cytoplasmic AID expression in these cells (Figure 31E and F) closely resembles the population of IF large B cells recently described in lymph nodes (5) where AID+ IF large B cells are mainly localised within the T cell area surrounding the follicles (196;695).

RA SYNOVIUM

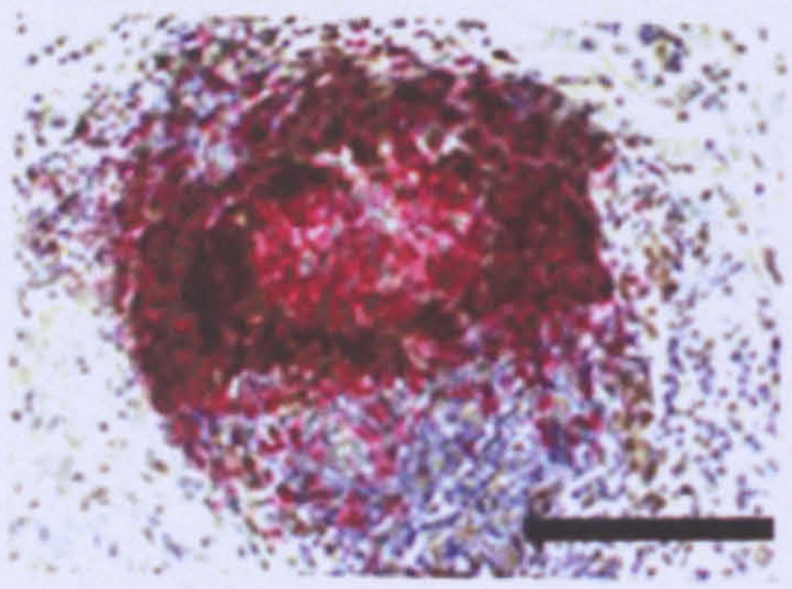
A. CD21/Bcl2



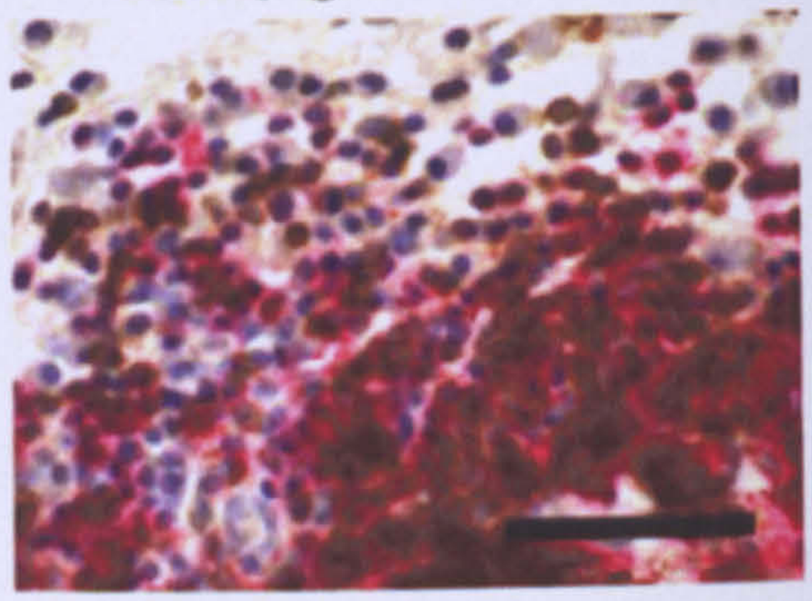
B. CD3



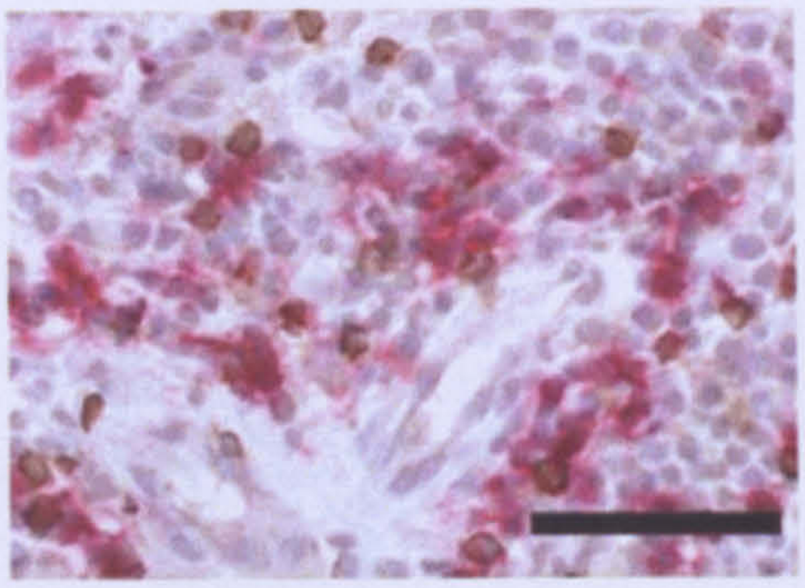
C. CD20/IgD



D. CD20/IgD



E. CD20/IgD



F. CD20/IgD

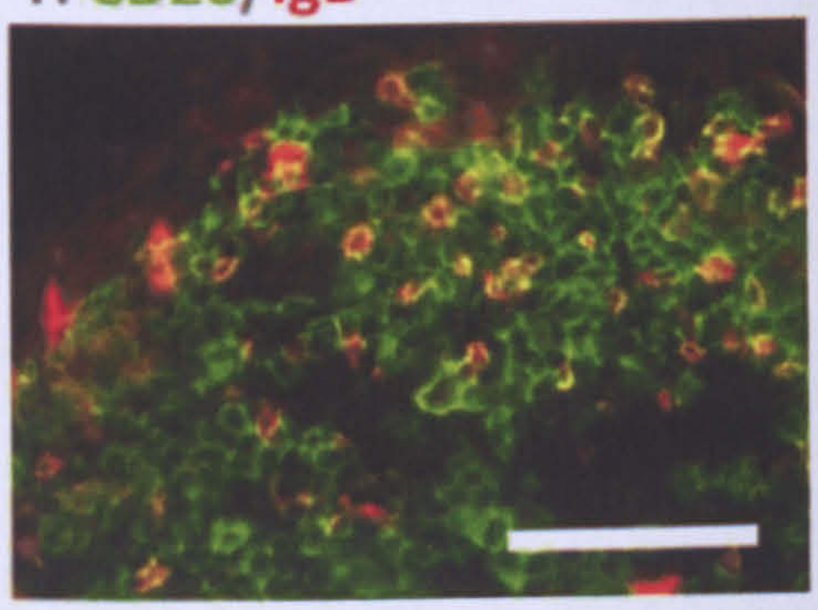
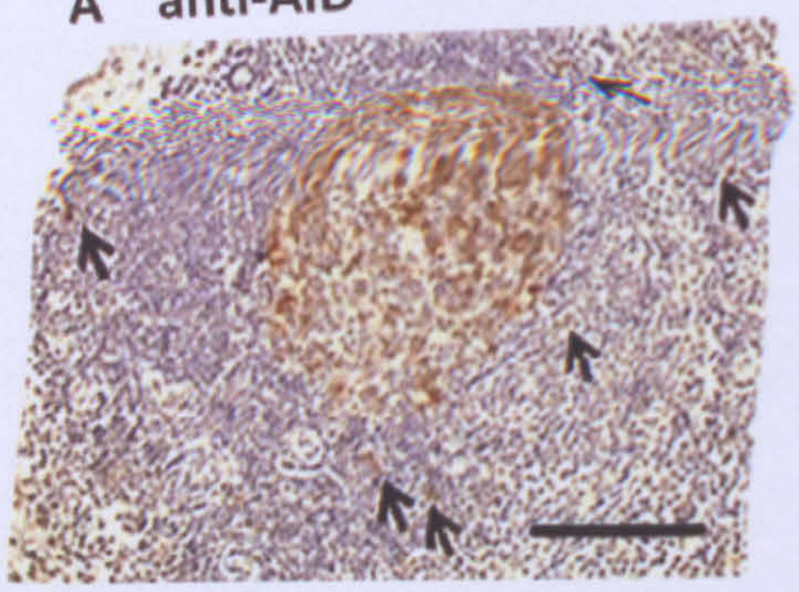


Figure 30: Ectopic germinal centre like structures within RA synovium are surrounded by large CD20+, IgD- B cells

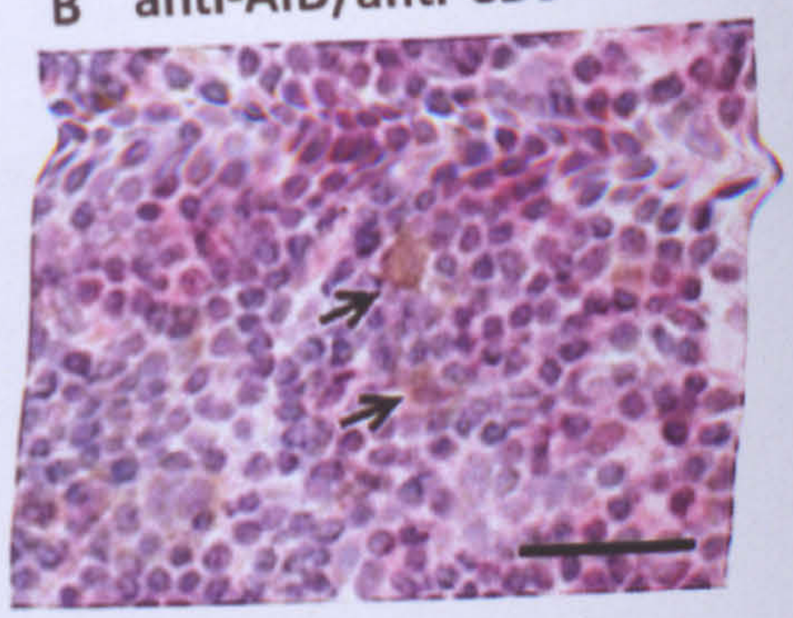
Sequential sections of paraffin embedded RA synovium were double stained with (A) CD21 (red) and Bcl2 (brown), (B) singly stained with CD3 and (C) double stained with CD20 (red) and IgD (brown) in order to identify tissues containing ectopic GC (original magnifications x20). (D) CD20+, IgD- cells were found surrounding ectopic GC within the T cell rich area (x40 magnification of C)(E) a subset of these cells morphologically resembled interfollicular large B cells with a large cytoplasm and dendritic like structure(x60 magnification of C). (F) In order to confirm single positivity for CD20, frozen sections of RA synovium underwent double staining using immunoflouresence for CD20 (green) and IgD (red). Scale Bars: 200μm (A,B, C), 100μm (D) 50μm (E,F)

RA SYNOVIUM

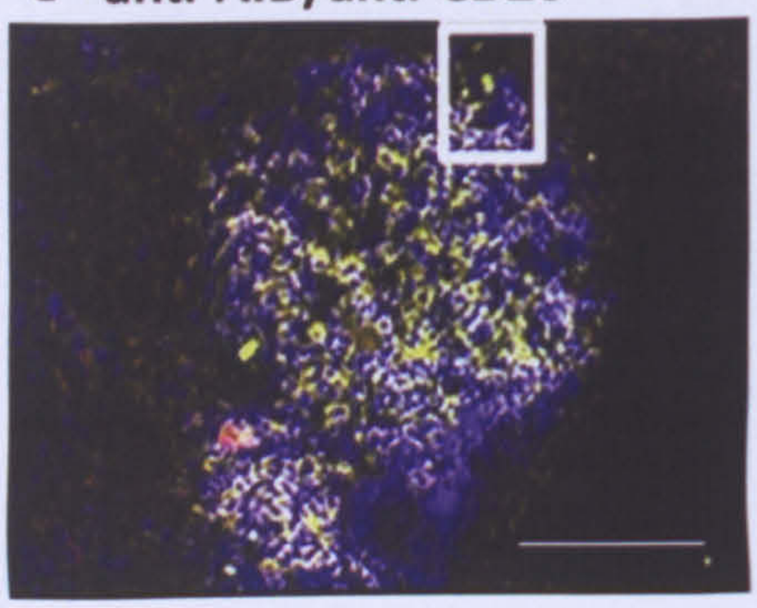
A anti-AID



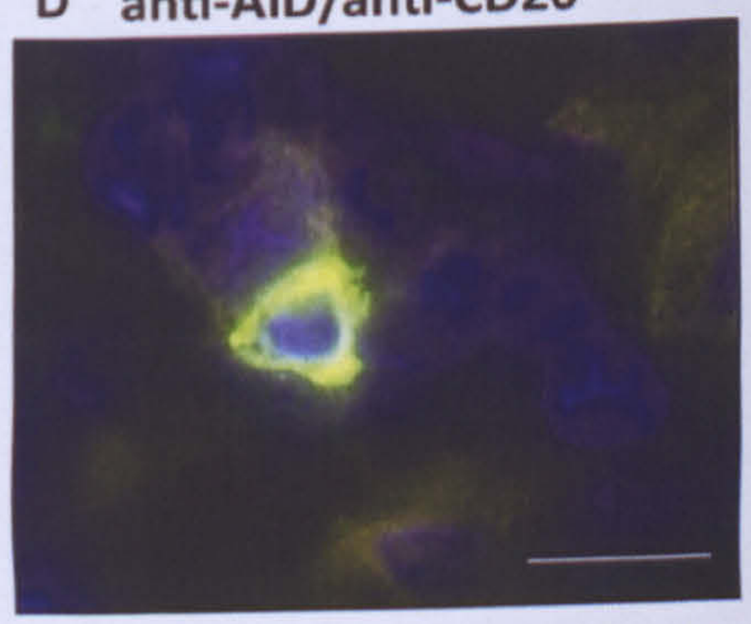
B anti-AID/anti-CD3



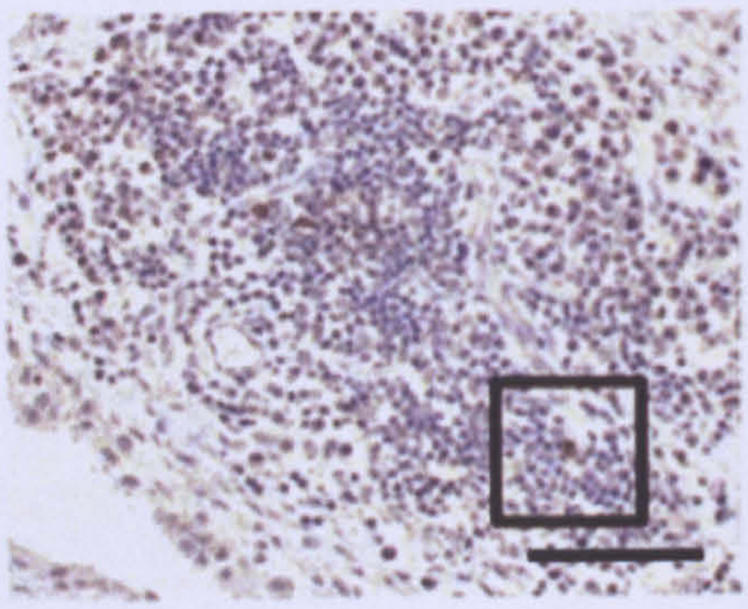
C anti-AID/anti-CD20



D anti-AID/anti-CD20



E anti-AID



F anti-AID

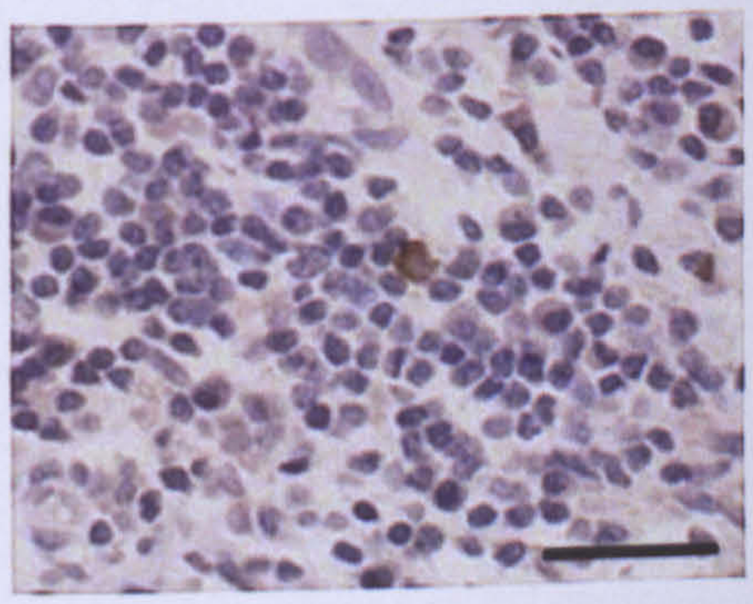


Figure 31: AID Identifies Interfollicular Large B cells within the Rheumatoid Synovial Membrane in Association with FDC-positive aggregates

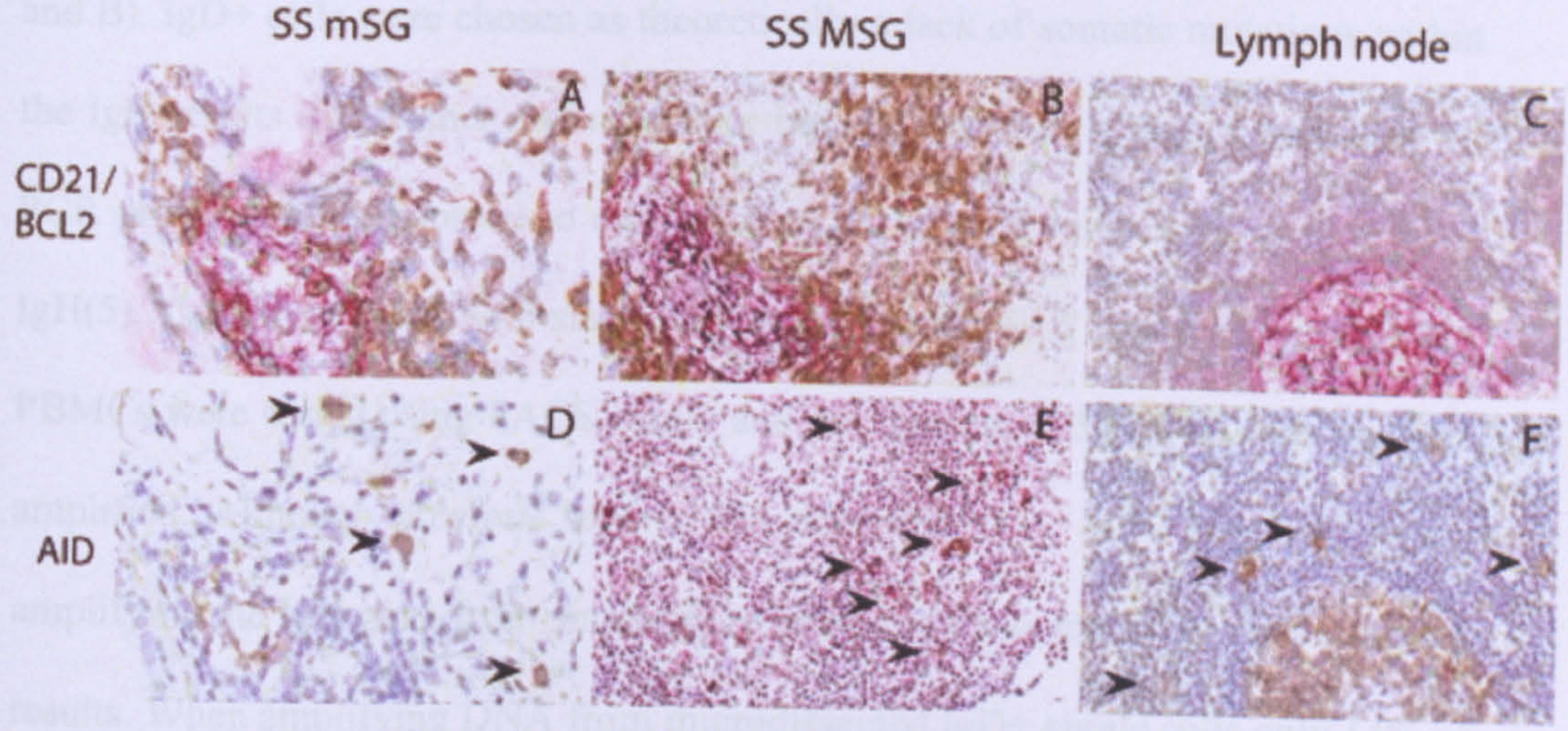
(A) Paraffin-embedded sections from RA patients were stained for AID (x 20 magnification). AID +ve cells with a large cytoplasm and sometimes dendritic like morphology (arrows) were frequently and exclusively seen in RA synovial tissue characterized by the presence of large aggregates predominantly in close proximity to CD21 +FDC networks. (B) Paraffin sections were double stained for AID (brown) and CD3 (red) (x60 magnification), demonstrating the close relationship between AID positive cells (arrows) and T cells in the peripheral T cell areas of the lymphoid aggregates. (C) Merged double staining for CD20 (green)/AID (red) on frozen RA sections confirmed that AID +ve cells were of B cell origin (double stained cells are identified in yellow) (D) Higher magnification of an example of an AID positive, CD20 positive cell (X60 magnification of (C)). Scattered AID positive cells (E) with the appearance of IF B cells (F) were occasionally found away from the central focus of the aggregate. Scale Bars: 200μm (A,C,E), 50μm (B,F) 15μm (D)

AID Expression Identifies IF Large B cells Surrounding Germinal Centres in Minor Salivary Glands and Parotids of Sjogren's Syndrome Patients

In order to test for disease specificity another chronically inflamed tissue, the salivary glands of patients with Sjogren's syndrome, was examined. In addition to GC B cells within FDC networks, a second pattern of AID expression was observed in minor salivary glands and parotids of SS patients. As shown in Figure 32, numerous large B cells often with dendritic morphology strongly expressed AID, mainly in the cytoplasm and to a lesser extent in the nucleus, and were localized in close association with CD21+AID+GCs in Sjogren's syndrome minor salivary glands (Figure 32, *A* and *D*) and Sjogren's syndrome parotids (Figure 32, *B* and *E*). Importantly, this population was exclusively observed in salivary glands where lymphocytic aggregates with CD21+ FDC networks were present.

Figure 32: AID expression in Sjogren's syndrome minor salivary glands and Sjogren's syndrome parotids also identifies cells resembling the recently described population of IF large B cells.

Sequential paraffin-embedded sections from Sjogren's syndrome patients were double stained for bcl-2 (brown) and CD21 (purple) (A-C) and single stained for AID (brown) (D-F). Representative examples of Sjogren's syndrome minor salivary glands (A-D) and parotids (B-E) are shown with a tertiary lymphoid follicle in a lymph node (C-F) for comparison. AID+ cells with a large cytoplasm and sometimes dendritic morphology (arrowheads, D-F) were frequently and exclusively observed in Sjogren's syndrome salivary glands characterised by the presence of large lymphocytic aggregates, often in close proximity with CD21+FDC networks (D,E). The morphology and pattern of distribution are highly reminiscent of the population of IF large B cells recently described in secondary lymphoid organs as shown in lymph node (F). (Original magnification, x200 in A-D, and x100 in B-E and C-F).


SS mSG
SS MSG
Lymph node
CD21/
BCL2
AID
A
B
C
D
E
F

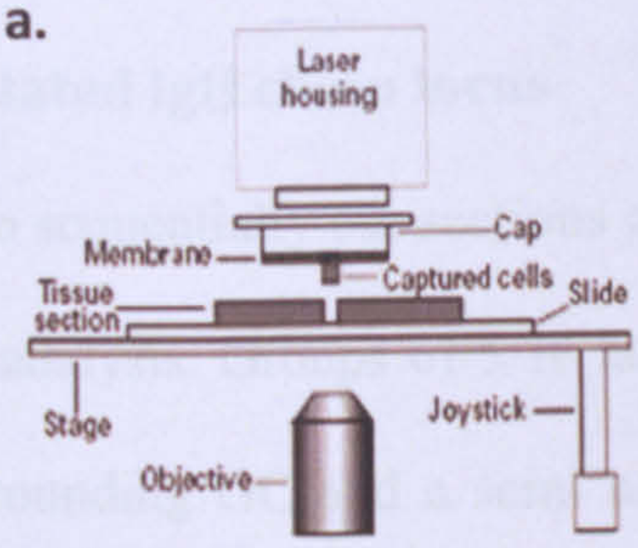
Single cell laser capture micro dissection reliably amplifies the IgH locus from groups of 5 IgD+ cells

In order to determine the efficiency of IgH amplification from singly dissected cells, IgD+ cells were microdissected from lymph node using LCM (Figure 33 A and B). IgD+ cells were chosen as theoretically a lack of somatic mutations within the IgH results in a higher rate of primer binding and thus a higher frequency of PCR product than compared to cells such as IF large B cells carrying a mutated IgH(5). Caps were filled with single, 5, 10 or 20 cells. As a positive control IgM+ PBMCs were sorted using FACS singly and into groups of 5 and 10 cells and IgH amplified. Although previous reports had documented a 60% efficiency when amplifying the IgH gene from single IgD+ cells (5) I was unable to replicate such results. When amplifying DNA from microdissected IgD+ single cells only 14% of PCR reactions resulted in a PCR product (Figure 33D lanes 2-8) vs 50% with single IgM+ sorted cells PBMCs and 70% with groups of 10 IgM+ cells (data not shown) . If 5 singly microdissected cells were grouped together however a PCR product was detected in approximately 83% (5/6) cases (Figure 33C lanes 3-8), and if 10 or 20 cells grouped together in a single cap 90% of PCR runs resulted in a positive PCR product (data not shown). Thus in order to both maximise the number of IgH genes amplified but to ensure only IF large AID+ cells were microdissected from tissue the experimental protocol was to micro dissect single cells but to group together 5 cells in each cap.

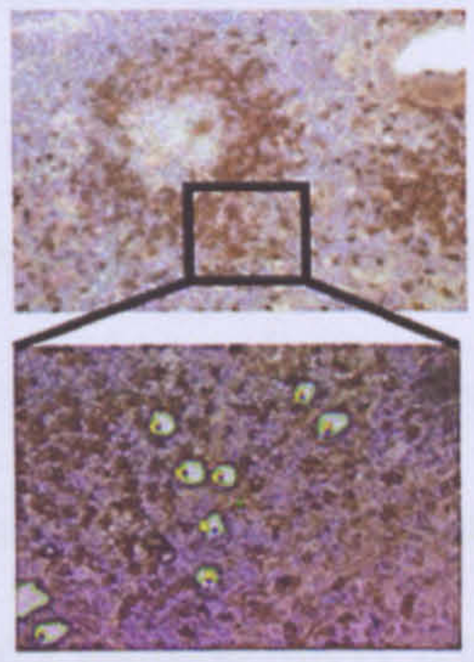
Figure 33: Single cell laser capture microdissection and semi nested PCR reliably amplifies the IgH locus from groups of 5 IgD+ cells

A laser microdissection microscope (PALM) (A) was used to microdissect single IgD positive cells from frozen sections of lymph node stained with IgD using immunohistochemistry (B) (original magnification x20 and inset x40). When 5 single microdissected cells were grouped together a PCR product was noted on 83% (5/6) of occasions (C), however when single cells were amplified a PCR product was noted only 14% (1/7) of times (D).

a.



b.



c.

1 PCR marker
2 T cells
3 -8 groups of 5 singly microdissected IgD+ cells

d.

1 PCR marker
2-8 single microdissected IgD+ cells

AID+ Interfollicular Large B cells from lymph node express a somatically mutated IgH chain locus

Two sequentially cut sections of reactive lymph node from 2 patients were chosen for analysis. Groups of 5 IF large B cells were microdissected from frozen tissue surrounding GC and a semi nested PCR performed for amplification of the IgH gene. Following cloning 65 colonies were sent for sequencing (LARK).

I was unable to microdissect AID+ IF large B cells from frozen RA synovial tissue. IF large B cells are seen less frequently within synovial tissue and the morphological integrity of frozen tissue samples is poorer than that of paraffin embedded tissue. These factors, together with poor quality immunohistochemical staining made attempts to singly microdissect IF large B cells using this approach unfeasible.

Results from lymph node are summarised in Table 19 and include a number of IgD+ cells sent for sequencing. A total of 28/65 AID+ colonies produced sequences, a number of these were repeated sequences, a result of sending an identical colony for sequencing. In addition of the 28 sequences obtained from IF large cells, 9 were unique sequences and of these 7 were mutated and two were unmutated. Results also demonstrated low level mutation within 1 IgD+ cell. The mean mutation rate for IF large B cells from the 2 lymph node samples examined was 3.3%(range 0-14.5%) which was lower than reported by Marafioti et al (mean 9% range 2-13%)(5).

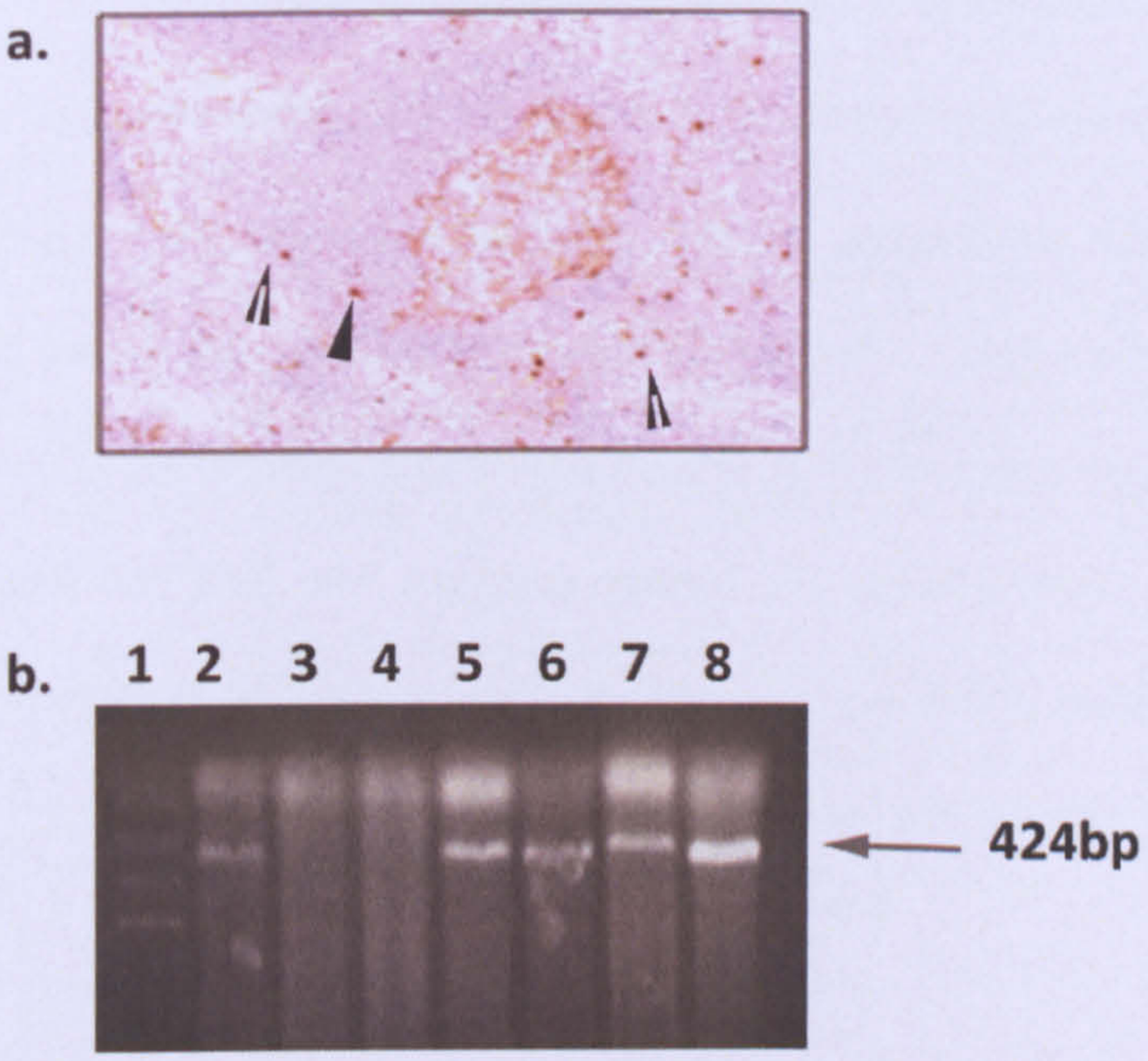


Figure 34; Single cell microdissection and amplification of IgH chain from interfollicular large B cells from lymph node

Sections of frozen lymph node underwent routine immunohistochemical staining for AID, arrow heads (A). Single cells were microdissected and the IgH gene from groups of 5 cells amplified using a semi-nested PCR. Positive PCR products (expected band size 424bp) were seen in this example from groups of 5 cells.

Table 19: Results of sequencing the IgH chain locus from interfollicular B cells microdissected from lymph node

The IgH chain locus from AID+ and IgD+ cells microdissected from lymph node was sent for sequencing. 68 stab colonies were sent from AID+ cells and 5 stab colonies from IgD+ cells. A number of sequences came back as nonsense and a number were repeated, thus overall only 9 sequences from AID+ cells and 2 from IgD cells were suitable for further analysis. The bold red line separates results from 2 lymph nodes from 2 patients. Cap code refers to sequences obtained from amplified cells within the same cap.

Cell type	Cap code	VH family	Clone number	Sequence summary	No mutations	D segment	J family	V Gene and family
AID +ve cells	B	VH3	Fi 37	Mutated	3	Short and complete	JH4	DP32 (VH3)
			Fi 82	mutated	6 (mostly ts to Cs)	X9051	JH3B	DP58 (VH3)
			Fi 88	Not mutated	0	Short and complete	JH3B	DP32 (VH3)
	D	VH3	Fi 69	mutated	2		JH 6	DP 353-11
	E	VH6	Fi 18	mutated	18	No matches	JH2	DP-15Z12317V1-8
	F	VH3	Fi 41	Mutated (heavily)	24 plus deletion		JH6B	DP 473
		VH6	Fj 7	Low level mutation	3	No matches	JH3B	VH6f
	Li 2	VH3	FH18	Highly mutated	44	No matches	JH5A	DP58 (VH3)
			FH19	Unmutated	0		JH3B	DP47-3 (VH3)
IgD cells		VH5	Fi127	Low level mutation	4	X97051	JH6A/B	DP-10Z12312
			FH45	Low level mutation	3	X97051	Jh5bf	DP-10z12312fdp

Discussion

Ectopic GCs within RA synovial tissue, capable of recapitulating many of the features of GCs found within secondary lymphoid organs have long been recognised(673). The micro architecture of these structures includes T/B cell separation and FDC networks(673). The identification of large CD20+, IgD- cells often with a dendritic like structure surrounding FDC+ aggregates within RA synovial tissue raised the possibility that these structures may also be capable of supporting IF large B cells, a novel subset identified within human peripheral lymphoid tissue(5) and recognised to uniquely express AID within the interfollicular area outside of the GC(196). Thus the identification of a cohort of CD20+, IgD- cells expressing AID outside of ectopic GC both in RA synovium and within the salivary glands of patient's with Sjogren's syndrome is the first report of IF large B cells at an ectopic site. Their characterisation within synovial tissue demonstrates a number of similarities with IF large B cells in secondary lymphoid organs, namely their localisation within the peripheral T cell area, a predominantly cytoplasmic staining pattern and a dendritic morphology. The role of IF large B cells is currently unknown, but their dendritic-like morphology and close association with T cells has led to speculation that they may play an important role as antigen presenting cells (5), although this is not supported by their lack of expression of costimulatory molecules such as CD80 and CD86 and only weak positivity for CD40(5). Interestingly, I also confirmed that the expression of IF large B cells is not limited to ectopic GCs within synovial tissue, and their identification in the salivary glands of patients with Sjögren's syndrome(675) suggests that these cells are associated with sites of ELN in the target organs of several autoimmune disorders.

Importantly, IF large B cells have also been proposed as non neoplastic precursors of B cell malignancies, including that of diffuse large B cell lymphomas, some of which harbour translocations generated by erroneous CSR(196). An association of RA with an increased risk of lymphoma has been recognised for some years, but only recently has a striking predominance of diffuse large B cell lymphoma been identified, particularly associated with increased RA disease activity(706). Thus the identification of IF large B cells within the synovial membrane in exclusive association with ectopic GCs, structures associated with both increased local and systemic inflammation(388) and further likely lacking normal immune regulatory mechanisms may provide an explanation for the excess of diffuse large B cell lymphoma seen within RA patients.

Although IF large B cells are recognised to express a somatically mutated IgH(5), initial suggestions that this may indicate a post GC B cell were questioned when around 10-20% of such cells were identified as expressing AID(196). This extrafollicular AID expression suggests that IF large B cells may represent an extrafollicular antibody response, with ongoing SHM occurring outside of the GC(196). Although AID activity within AID+ IF large B cells has been confirmed previously by the detection of circle transcripts, evidence for a somatically mutated IgH was not specifically examined. By micro dissecting only AID + IF large B cells from lymph node I can however confirm the earlier report that these cells have a somatically mutated IgH, although at a much lower level than previously reported (3.3 vs 9%). There are a number of reasons for this discrepancy in levels of SHM. Firstly, although the initial report of somatically mutated IF large B cells used single cell dissection, a technique of micromanipulation (710) was utilised rather than LCM. In addition double

immunohistochemistry was used to identify IF large B cells prior to isolation rather than the use of a single marker, inherently more distinctive used within this study. Both these approaches likely make the isolation of pure IF large B cells technically more difficult. Further, as only a cohort of IF large B cells express AID (10-20%)(196), the identification of IF large B cells using other markers is likely to include both AID+ and AID- cells. It could therefore be hypothesised that those IF large B cells that do not express AID have already undergone a process of SHM, whereas those cells expressing AID are actively mutating their IgH, thus micro dissecting AID + cells would result in the isolation of cells with a inherently less mutated IgH. In addition when establishing the technique of LCM and amplification of the IgH within our laboratory I was unable to reproduce the previously reported sensitivity of 60% when amplifying the IgH locus from IgD+ cells, with a sensitivity of only 10% when using a semi-nested PCR. Thus in order to increase the chance of micro dissecting a whole nucleus (in general around 5μm in diameter) I used 10μm thick tissue sections. However, the use of sections with an increased diameter does increase the risk of including DNA not from the cell of interest, such as naive B cells, that may carry a non mutated IgH. This additionally may explain my result of low level SHM in a single IgD+ cell. Furthermore, although IgD+ cells surrounding GCs are theoretically naive B cells and thus carry an unmutated IgH, there are reports of infrequent IgD+ B cells carrying a somatically mutated IgH(711).

Finally, attempts to microdissect single IF large B cells from RA synovial tissue were not successful due to a number of problems. Firstly as IF large B cells are found in much reduced number within RA synovial tissue, morphological integrity of synovial tissue and high quality of IHC staining for AID was essential in order

to resolve and thus microdissect single IF large B cells within synovial tissue. Due to both the quality of frozen synovial tissue available and the poor results of IHC staining on frozen RA samples single cell micro dissection was not feasible. Although it is possible to amplify the IgH from paraffin embedded tissues (using primers for the FR3 region of IgH), the specialised membrane covered slides required for LCM do not tolerate heat based antigen retrieval techniques required for AID staining on paraffin tissue and the AID staining protocol could not be optimised with other enzyme based antigen retrieval techniques (including trypsin and proteinase K). Membrane covered slides suitable for heat based antigen retrieval are currently under development with PALM and when commercially available the single cell micro dissection of IF large B cells from RA synovial tissue could be undertaken.

In summary I present the novel finding that IF large B cells can be identified within RA synovial tissue in exclusive association with FDC+ lymphocytic aggregates, their identification in the salivary glands of Sjogren's syndrome patients further suggests they may be found in ectopic sites in a number of autoimmune disorders. I also demonstrate that AID+ IF B cells in lymph node express a somatically mutated IgH. Further work needs to investigate whether IF large B cells within RA synovial tissue express a somatically mutated IgH and a study of expression patterns of dendritic cell markers and co stimulatory molecules will aid in elucidating the function of these cells.

Chapter 7: General discussion

The presence of ectopic GCs within RA synovial tissue has been recognised for some time, although controversy has surrounded whether their detection defines a poorer prognostic category and further whether they are themselves directly pathogenic(426).

As current prognostic markers are unable to reliably predict those RA patients most at risk of rapid disease progression and thus direct the most intensive therapeutic interventions to those patients in greatest need, additional prognostic markers are urgently needed. Previous work has suggested that histological classification of synovial tissue into a diffuse, aggregate or GC + infiltrate associates with clinical phenotype (217;421). Such a mutually division is not reflected however on examination of synovial tissue from other patient cohorts, with a frequent overlap of all 3 subdivisions(388). Furthermore, no account is made of the degree of diffuse or aggregate tendency of tissue. The first major component of this thesis therefore, sought to develop and validate a quantitative aggregational scoring system for RA synovium using DIA that quantified both the degree of aggregation and diffuse infiltration within synovial tissue. It further aimed to examine both whether the presence of ELN or the degree of aggregation/diffuse infiltration correlated with clinical phenotype and with joint damage progression.

I am able to report a number of observations:

- Firstly that the novel quantitative aggregational score developed is a highly reliable scoring system
- Secondly that ELN associates with synovial inflammation, and the degree of aggregation (AS) and overall cellular infiltrate (DS) within the synovial membrane correlates with systemic inflammation

- Thirdly, that there is no association between the presence of ELN within synovial tissue and the presence of serum autoantibodies, and
- Finally, that neither the degree of aggregation nor diffuse infiltrate predicted joint damage progression over a 2 year period

The demonstration that the quantitative aggregational scoring system developed is highly reliable suggests a high degree of sensitivity to detect change within the synovial membrane and thus suggests potential long term application to both clinical studies and drug trials. However this study also demonstrated significant variability in both diffuse and aggregational tendency within the joint. How much synovial tissue to sample to detect reliably a specific parameter has been investigated by a number of studies, and indeed consensus opinion is that the examination of six synovial biopsies is sufficient to eliminate significant variability(674). Whether this applies to the detection of aggregates has so far not been specifically addressed and in particular whether sampling of the joint from one site (for example from the SPP) is adequate is not known, and indeed is not supported by My results. This consideration is particularly important when considering the results of this study demonstrating a lack of association found with serum autoantibodies, a result reported by a number of other groups and taken to imply lack of functionality of such structures(416). It is likely however that ectopic GC form asynchronously and/or heterogeneously in different joints or areas within the same joint and thus such structures could easily be missed by limited sampling of a single site or by limited biopsies from different sites. Thus a specific study powered to determine the variation in aggregation within the joint is required in order to determine the number of biopsies and sites required to reliably categorise a patient as diffuse or aggregate.

A further limitation of this study is addressing correlations with clinical and biochemical markers on a historical cohort of patients with varying disease duration and on a number of DMARDs. Although this was the first study to investigate whether the aggregational tendency of synovial tissue predicted joint damage progression (rather than associate with erosive damage in a cross sectional study), it is known in particular that DMARDs are capable of modifying the histological appearance of the synovial membrane(712). In addition the long term aim of novel biomarkers is their use in early arthritis to predict early on which patients require the most intensive therapeutic interventions (including biologics). Therefore the optimum study to investigate the prognostic value of the aggregational tendency of synovial tissue to predict joint damage progression would recruit patients with early RA, naive to all therapy, a study that is currently underway within our group. Indeed there is already evidence suggesting that lymphoid neogenesis may relate to specific clinical-biological behaviours when patients are prospectively analyzed. Kraan et al. (713) in a 2-year follow-up study observed that high scores for B cell, plasma cell, and macrophage infiltration evaluated in early stages (n = 95) were the best discriminating markers in predicting the diagnosis of RA versus other forms of arthritis. Further, Canete et al.(387) demonstrated that the presence of lymphoid aggregates plus T–B cell segregation and PNAd+ HEV, assessed at baseline in a cohort of 86 patients, is a highly significant negative predictor of moderate-good EULAR response to anti-TNF therapy.

Importantly as stated although I demonstrated a lack of an association between aggregational tendency of the synovial membrane and autoantibodies within the serum of patients I did not take this to imply lack of functionality of such

structures. The lack of an association may not only be a result of limited sampling being unable to reflect the histological picture of the whole joint, but the production of autoantibodies including RF and ACPA has been demonstrated to occur at other distant ectopic sites such as the lung, complicating the relationship between detection in the sera and local production within synovial tissue. Whether ectopic GCs are indeed functional or merely by products of inflammation has created much debate in the literature(416;424;426), and is a crucial question to address in order to understand the pathogenic mechanisms driving local inflammation and autoimmunity within the synovial membrane. Thus the second main component of this thesis investigated whether ectopic lymphoid structures within RA synovial tissue expressed the molecular machinery required for the production of high affinity class switched antibodies and further by transplanting synovial tissue into SCID mice, whether such structures were actively involved in the pathogenic process via the production of potentially pathogenic autoantibodies. This work resulted in a number of important observations in particular:

- At both mRNA and protein level AID is expressed within RA synovium in exclusive association with lymphocytic aggregates expressing FDCs
- That AID expression is maintained within synovial grafts transplanted into SCID mice up to 4 weeks post transplantation and associated with the detection of human IgG anti-CCP antibodies in mouse sera;
- That AID expression is functional, as determined by the detection of Iγ-Cμ circular transcripts (a marker of ongoing CSR) in AID+ RA-synovium and grafts;

- That AID is associated with high levels of molecules regulating the development and maintenance of ectopic lymphoneogenesis (CXCL13, TNFα, LTβ) and the B-cell proliferating factor APRIL.

This work demonstrates the potential of the autonomous capacity of the RA-synovium to sustain B-cell growth, maturation and differentiation suggesting a pathogenic role for ectopic lymphoid structures in disease relapse/resistance after rituximab therapy.

This unique model enabled the detection of autoantibodies in tissues transplanted into SCID animals in the absence of confounding factors, such as their possible production in secondary lymphoid organs or the migration of new B and plasma cells to the grafted tissues. By transplanting RA synovial tissue with and without lymphoid aggregates into SCID mice, I demonstrated that AID+CD21L+ RA synovial grafts produce high levels of human IgG ACPAs, which can be measured in the mouse circulation, and whose titres are associated with the expression levels of AID mRNA in the synovial grafts. Of relevance, production of ACPA by synovial grafts persisted several weeks after transplantation, and was associated with persistent proliferation of B cells, and concomitant detection of Iγ-Cμ circular transcripts, suggesting that TLS can in part behave as self-sustained microanatomical immunological units capable of supporting functional AID expression and IgM to IgG class switching independently from the systemic circulation. In keeping with these observations, I also demonstrated that ectopic lymphoid follicles in RA patients can be surrounded by CD138+ plasma cells producing antibodies directed against citrullinated fibrinogen.

Collectively, these data indicate that in addition to accumulation from the periphery, autoreactive plasma cells can be supported in situ by AID+ lymphoid

aggregates, suggesting that they may function as 'protective niches' for local B-cell differentiation (Figure 35) and auto-antibody production with the potential of directly contributing to the autoimmune and response. Expanding on this evidence I have recently proposed a working model for lymphoid tissue response and autoantibody production in RA (Figure 36)(6) in which a sensitization phase would take place outside the joint following an environmental insult [i.e. citrullination of lung proteins in smokers (110) in association with other predisposing risk factors (i.e. HLA shared epitope), resulting in presentation of auto-immune epitopes and leading to T- and B-cell priming in secondary lymphoid tissues. B-cell sensitization leads to the production of auto-antibodies followed by a period of latency that could last years. A second systemic 'hit' related to unknown triggers (possibly infection) could then lead to specific or polyclonal B-cell activation with a rise in circulating antibodies and immune complex (IC) formation(714). IC activation of FcRs in the portal circulation results in the secretion of systemic vasoactive amines in a mechanism that would have a selective effect on endothelial permeability. The distinct vascular properties of synovial endothelium would result in a preferential extravasation of macromolecules and auto-antibodies to the joint, potentially explaining why arthritis accompanies many human infectious and autoimmune disorders(715). Again subsequent unmasking of neo-epitopes (such as citrullinated proteins) within synovial tissue would lead to local pathology. Thus it is possible that such tissue damage would promote further exposure of synovial antigens, also supported by spreading to unrelated epitopes and in situ presentation of cryptic determinants (716;717). This would lead a persistent immunostimulation, triggering, in some individuals or disease stages, the described mechanisms of

lymphoneogenesis, and the consequent modulation of the humoral response, supported by transient developmental cycles or persistent localization of newly formed tertiary lymphoid structures (Figure 36).

Further, a crucial question leading on from this work is whether response-resistance to rituximab treatment in RA patients is defined by i) distinct cellular and molecular phenotypes within RA-synovium and ii) whether response-resistance-relapse to rituximab is determined by B-cell depletion and/or survival (in "protected" self-driven synovial niches). This study is currently ongoing in the department, recruiting RA patients resistant to anti-TNF therapy for pre and post rituximab minimally invasive ultrasound guided synovial biopsies. Following histopathological characterisation and gene expression analysis of synovial tissue critical pathways involved in B-cell survival, activation and repopulation will therefore be determined.

In addition this work crucially validates the SCID-HuRA chimera as a translational tool to investigate/modulate B-cell function and following the identification of novel pathways involved in B cell activation and survival identified within the synovial membrane pre and post rituximab I will also have the opportunity to target these pathways in the Hu-RA SCID mouse model. This will permit proof of concept studies to be performed using a variety of systems including intra-graft injection of monoclonal Ab (e.g. anti-BAFF), fusion proteins (e.g. TACI-Ig, CTLA4-Ig), siRNA and dominant negative mutants in adenoviral vectors as appropriate. Biological readouts will include graft modulation of: a) BAFF, APRIL, PBEF and TSLP as detected by IHC and QT-PCR; b) survival and proliferation of synovial B-cells and IHC analysis of ectopic lymphoid aggregates; c) AID expression and ongoing CSR; and d) human class-switched anti-CCP

antibodies. This work is critical as one limitation of My study is a lack of a direct link between AID+ lymphoid structures and anti-CCP antibody production, evidence that could be provided in the SCID-HuRA if anti-CCP antibody production is abrogated, for example, following the disruption of FDC networks with compounds such as LTβR-Ig or TACI-Ig. Thus the potential for this ongoing work is to advance knowledge of the mechanisms regulating B-cell activation, AID expression and ectopic antibody formation and subsequently translate these new observations back to RA patients. Indeed a number of recent studies focusing on the synovial membrane's response to specific biologic agents (387;389;680;718) have resulted in a number of important observations regarding the role of specific immunological pathways related to B cell activation within target tissues in disease pathogenesis. A number of other biologic agents with the potential to interfere with lymphoid tissue response are in clinical development (Table 20) and future data examining their effect on the synovial membrane and lymph node will undoubtedly lead to further advancements regarding the pathogenic role of these compartments during early, established and advanced stages of the disease.

Finally, a further important question remains unanswered despite important work in this area, that of the specific antigen driving the production of ectopic GCs within RA synovial tissue. This is a crucial question to address to not only further the understanding of RA pathogenesis but in addition to develop novel treatment approaches for RA such as the induction of tolerance. Thus further experiments are planned to identify not only the antigen specificity of B cells within ectopic GCs within synovial tissues but also antigen specificities within GCs within locally draining lymph nodes. This line of investigation will allow us to identify whether

the immune response within the synovial membrane becomes uncoupled from that in local draining lymph node. Understanding the relationship between B-cell activation in the synovium and local lymph node is crucial to enable therapeutic targeting of the primary site of initiation and maintenance of the disease process.

The third main component of this thesis aimed to investigate whether a cohort of large CD20+, IgD-ve cells surrounding ectopic GCs were IF large B cells and whether AID+ interfollicular B cells, both within lymph node and the synovial membrane, expressed somatically hypermutated IgH genes. I report the following novel findings;

- Firstly I demonstrate that IF large B cells are found within synovial tissue, in exclusive associated with FDC+ lymphoid aggregates, and
- That IF large B cells within lymph node express a somatically mutated IgH gene.

My finding of IF large B cells within both rheumatoid synovial tissue and within the salivary glands of patients with Sjogren's syndrome suggests that such cells may be found in ectopic lymphoid tissue of a number of auto inflammatory conditions. Although I demonstrate that IF large B cells at ectopic sites share a number of similarities with those reported in lymph node and tonsil, such as location within the T cell rich interfollicular area, a close association with T cells, and a dendritic morphology, the next steps are to identify their functional role. A number of molecular markers may aid in addressing this question such as the expression of MHC class II markers and costimulatory molecules, if indeed they do play a role in antigen presentation, autoantibody expression (including RF and anti-CCP) and importantly the relationship of their IgH gene to GC B cells within

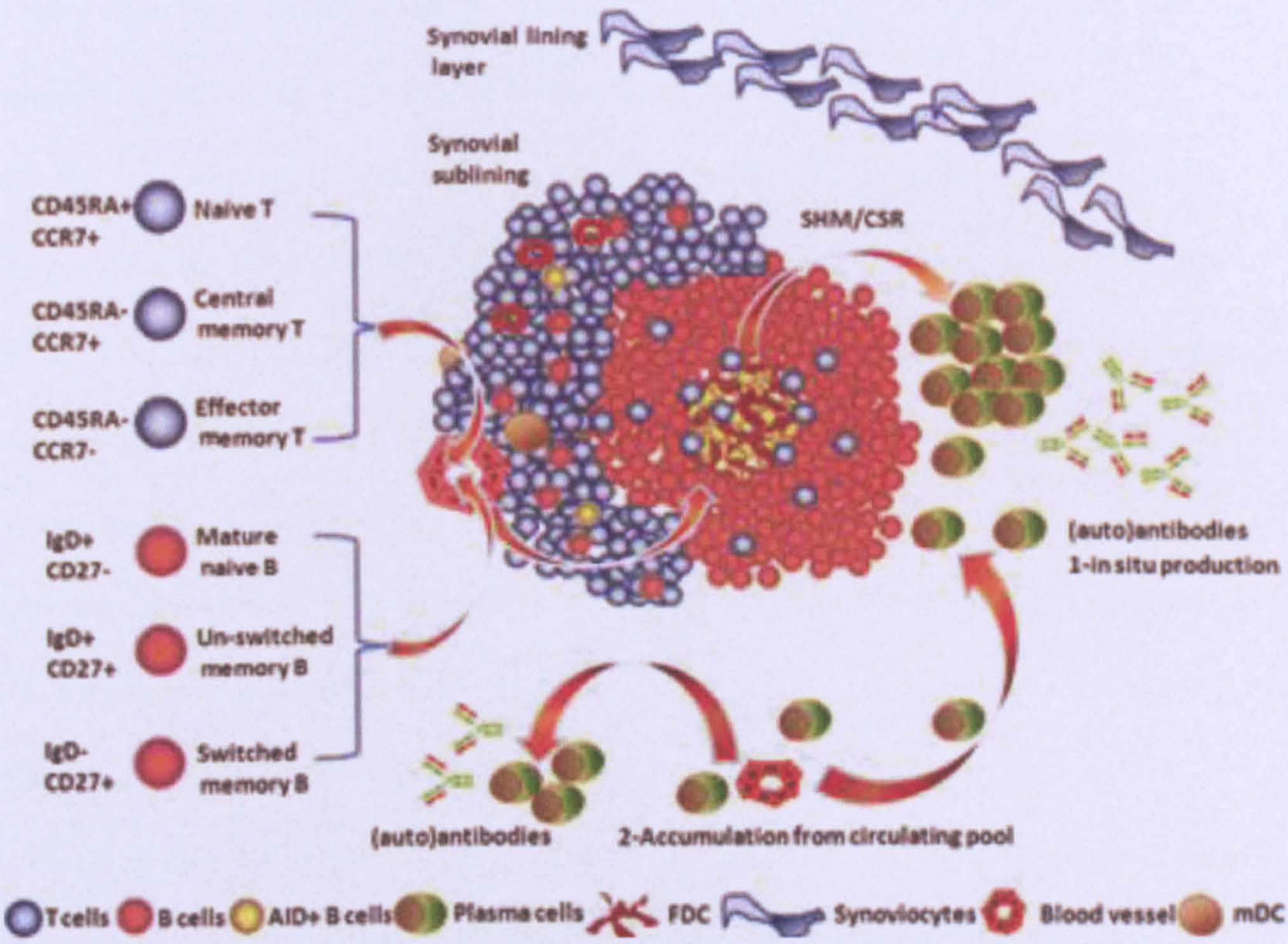
the local GC. A weakness of our study was the inability to microdissect IF large B cells from synovial tissue, however these technical issues are likely to be addressed imminently and thus the relationship of IF large B cells to the nearby GC could be addressed both in secondary and ectopic lymphoid tissue. Crucially understanding this relationship will allow us to address whether IF large B cells originate from the GC (and thus express a IgH in common) or whether they are an independent site of extrafollicular antibody production.

Understanding the functional role of IF large B cells has a number of important implications for understanding RA pathogenesis. Firstly, are these cells GC founder cells? If so could such cells be directly targeted to abrogate the local immune response within synovial tissue? Secondly, do these cells play a role in the extrafollicular production of autoantibodies? Thirdly, a link has been suggested between IF large B cells and diffuse large B cell lymphoma(196), recently identified as the predominant lymphomatous subtype in RA patients(706). It may be suggested therefore that such an excess of diffuse large B cell lymphoma in RA patients arises because of the aberrant development of IF large B cells within ectopic lymphoid tissue, at a site lacking normal regulatory immune mechanisms.

In summary, the present thesis provides evidence to support a direct role for ELN in the pathogenesis of RA, linking its presence to increased synovial inflammation and suggesting a direct pathogenic role via the production of autoantibodies.

Figure 35: Functional T/ B cells aggregates with FDC networks support (auto)antibody repertoire diversification and isotype class switching in B cells infiltrating rheumatoid synovial membrane.

Proposed schematic model of (auto) reactive B-cell activation within RA synovium. During chronic RA synovitis, different B-cell and T-cell subsets have been shown to be recruited to the inflammatory site. Perivascular lymphocytic aggregates developing in the synovial sublining can acquire features of secondary lymphoid organs such as FDC networks which support expression of activation-induced cytidine deaminase (AID) in B lymphocytes within these structures. In this environment B cells undergo Ig repertoire diversification and affinity maturation via rounds of somatic hypermutation (SHM) and isotype class switching via CSR in a germinal centre-like humoral response (1-in situ production of (auto)antibodies). In parallel (or in some RA patients, alternatively), plasma cells generated in other locations may also be recruited from the systemic circulation (2-accumulation of (auto)antibody-producing cells from the periphery) and be released into synovial environment where they can survive as terminally differentiated long-lived plasma cells. It still uncertain whether ectopic lymphoid structures can support the 'de novo' recruitment of naive T and B cells or whether memory cells undergo cycles of amplification within these micro-immunological units. mDC, mature dendritic cells. Reproduced from (6)


Synovial lining layer
Synovial sublining
SHM/CSR
CD45RA+ CCR7+
Naive T
CD45RA- CCR7+
Central memory T
CD45RA- CCR7-
Effector memory T
IgD+ CD27-
Mature naive B
IgD+ CD27+
Un-switched memory B
IgD- CD27+
Switched memory B
(auto)antibodies 1-in situ production
(auto)antibodies
2-Accumulation from circulating pool
T cells
B cells
AID+ B cells
Plasma cells
FDC
Synoviocytes
Blood vessel
mDC

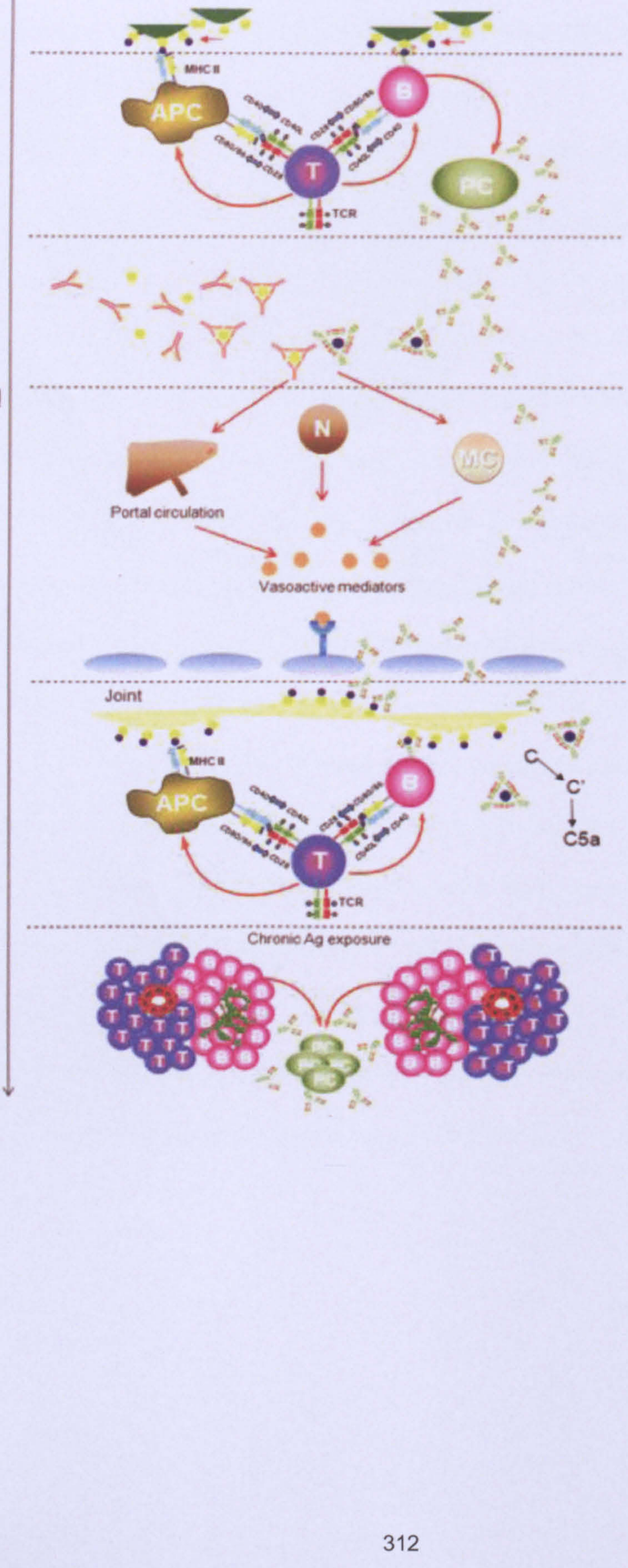

Time
MHC II
APC
B
T
PC
TCR
N
MC
Portal circulation
Vasoactive mediators
Joint
C
C'
C5a
Chronic Ag exposure

Figure 36: From extra-synovial sensitization triggered by non-specific antigen(s) to joint-specific inflammation in rheumatoid synovium: from increased vascular permeability to ectopic lymphoid tissue formation.

(A) An environmental insult (i.e. citrullination of lung proteins in smokers) in association with other risk factors (i.e. HLA shared epitope) results in modification of amino acid residues leading to antigen presentation and priming. (B) Antigen presenting cell (APC) and T-cell dependent B-cell sensitization and production of autoantibodies is followed by a period of latency. (C) A 'second hit' resulting in polyclonal B-cell activation (i.e. viral infection), induces rise in circulating antibodies and immune complex formation. (D) Activation of FcRs in the portal circulation and possibly mast cells results in the secretion of systemic vasoactive amines in a mechanism also dependent on neutrophils. These have a selective effect on endothelial permeability to macromolecules in the joints with extravasation of autoantibodies. (E) Resulting non-specific inflammatory process leads to unmasking of novel joint specific antigenic epitopes (i.e. citrullination of synovial proteins) and local pathology. (F) Chronic antigenic exposure within synovial tissue results in persistent immune stimulation triggering the described mechanisms of TLS formation and the consequent local modulation of the immune-inflammatory process. PC, plasma cells; N, neutrophils, MC, mast cells; C, complement. Reproduced from(6).

Developmental Stage	Agent (s)	Mechanism of action
Licensed for use in RA	Rituximab (Chimeric anti-CD20 Mab)	B cell depletion
	Abatacept (CTLA4-Ig)	Disruption of T cell co stimulatory pathways
	Infliximab, adalimumab (anti-TNF-α Mabs)	Blockade of TNFα
	Etanercept (TNFR-Ig)	Blockade of LT/TNFα
In clinical development	ocrelizumab, ofatumumab (humanised anti-CD20 Mabs)	B cell depletion
	TRU-015 (SMIP anti-CD20)	B cell depletion
	Belimumab (Humanised anti-BAFF Mab)	Inhibit B cell development, activation and survival via blockade of BAFF
	AMG 623 (BR3–Ig/BR3-Fc)	BAFF blockade
	Anti-BR3 Mab	BAFF blockade
	Atacicept (TACI–Ig)	Inhibit B cell activation, development and survival via blockade of BAFF/APRIL
	Baminercept (LTβR–Ig)	Disruption of GC formation and survival via LTβ blockade

SMIP, small modular immunopharmaceutical protein; GC, germinal centre; Mab, monoclonal antibody. Modified from ((719))

Table 20: Summary of therapeutic agents both licensed and undergoing clinical trial with the potential to modulate lymphoid tissue response

APPENDICES

IMMUNOHISTOCHEMICAL REAGENTS

TBS buffer 10x pH 7.6

60.6g Trizma HCL

13.9g Trizma base

90g NaCl

Make up to 1L with distilled water

Tris/HCL pH 8.2

24.22g Tris Base to IL of distilled water

Vector Red

To 4.75ml of TrisHCl (pH 8.2) add 0.25mls of levamisole, followed by 2 drops of reagent 1, 2 drops of reagent 2 and 2 drops of reagent 3. Mix well following each addition. Working solution should be used immediately.

1% Acid alcohol

Levamisole

1mM levamisole solution was prepared from stock solution 20x (Invitrogen)

Avidin-Biotin Complex (ABC) (DAKO K0355)

To make up solution add 10µl of solution A to 1ml of TBS (x1), mix well then add 10µl of solution B and incubate for at least 30 mins prior to use

REAGENTS FOR MOLECULAR BIOLOGY

1x TBE(Tris borate EDTA) buffer (0.089M TRIS, 0.089M Borate, 0.002M EDTA)

IL of concentrate (VWR EM8800) was made up to 5L with dH_20

Agar

Yeast extract 2g

Agar 6g

NaCl 2g

Bact tryptone 4g

And make up to 400mls with distilled water

LB/ampicillin/IPTG/X-Gal for plates

To 400ml of agar add: 800µl of ampicillin (50mg/ml) (Sigma A9518)

2mls IPTG (0.1M) (Promega V3955)

640µl Xgal (50mg/ml) (Promega V3941)

Publications Arising Directly From This Thesis

1. Secondary and Ectopic Lymphoid Tissue Responses in Rheumatoid Arthritis: from Inflammation to Autoimmunity and Tissue Damage/Remodelling. *Manzo, A., Bombardieri, M.,* ***Humby, F****, Pitzalis, C.,* Immunological Reviews, 2010 Jan vol 233 (1), 267-285. (Review Article)
2. Ectopic Lymphoid Structures Expressing AID Drive Self Sustained in situ Production of Autoantibodies in Rheumatoid Synovium ***F. Humby****, M. Bombardieri, A. Manzo, M. Blades, B. Kirkham, J. Spencer and C. Pitzalis.* PLoS Med 2009 Jan 13;6(1)
3. Animal Models of Rheumatoid Arthritis, *Patel HB, Dawson B,* ***Humby F****, Blades M, Pitzalis C, Burnet M, Seed M* Chapter 30, 385-412. Fundamentals of Inflammation, Ed Charles N. Serhan, Peter A. Ward and Derek W. Gilroy Cambridge University Press, 2009 (Book Chapter)
4. Activation-induced cytidine deaminase expression in follicular dendritic cell networks and interfollicular large B cells supports functionality of ectopic lymphoneogenesis in autoimmune sialoadenitis and MALT lymphoma in Sjogrens syndrome *M Bombardieri, F Barone,* ***F Humby****, S Kelly, M McGurk, P Morgan, S Challacombe, S de Vita, G Valensini, J Spencer and C Pitzalis* J Immunol. 2007 Oct 1;179 (7):4929-4938
5. The synovial membrane as a prognostic marker in rheumatoid arthritis, ***F Humby****, A Manzo, B Kirkham and C Pitzalis* Autoimm Rev 6:(4), 248-52 March 2007 (Review article)
6. Chemokines in Arthritis, ***F Humby****, A Manzo and C Pitzalis*. Future Rheumatology, February 2006 vol 1 No 1 (Review article)

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